L-Proline sensor based on layer-by-layer immobilization of thermostable dye-linked L-proline dehydrogenase and polymerized mediator

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Abstract

L-Proline sensor has been fabricated through multilayer assembly of recombinant dye-linked L-proline dehydrogenase (L-proDH) originally from hyperthermophilic archaeon (Therococcus profundus) and polymerized mediator, poly(allylamine) ferrocene (PAA-Fc), on a gold electrode by electrostatic layer-by-layer alternate adsorption method. The characteristic redox peaks of ferrocene groups were obvious exhibited in cyclic voltammograms, and both anodic and cathodic peaks increased with PAA-Fc/L-proDH bilayer number which confirmed the formation of a multilayer structure. Further experiments showed that an electrode fabricated in this way catalyzed the oxidation of L-proline. It suggested that polymerized mediator could transfer electrons between electrode surface and immobilized L-proDH. Amperometric experiments showed that the current response to L-proline increased with PAA-Fc/L-proDH bilayer number. The stability of the sensor was measured, and it kept a 40% relative response after 30 days storage in buffer under 4 °C.

Keywords: Layer-by-layer adsorption (LBL); Dye-linked L-proline dehydrogenase; Polymerized mediator; L-Proline sensor

1. Introduction

As an essential component, L-proline is important for proper functioning of joints and tendons. It not only implicates some disorders in the human body, but is also a practical subject in foods and pharmaceutical industries [1,2]. Several methods for L-proline determination have been developed and reported. They were based on fluorescence [3], chromatography [4–6] and electrochemiluminescence [7]. Although, these methods can determine the substrate in a relatively sensitive scale, chromatography and fluorescence methods always require a long time for analysis and pre-treatment of sample. The disadvantages of chemiluminescence include expensive and toxic reagents and interferences from impurities in real samples.

As a simple and sensitive method, electrochemical detection has been developed for the determination of amino acids. For example, biosensors incorporating broad-spectrum amino acid oxidase (AAO) with electrochemical transducer were utilized in the construction of amperometric biosensors for the determination of various kinds of substrates with high sensitivity and rapid analytical speed [8,9]. Furthermore, biosensors fabricated by immobilizing specific amino acid enzyme have also been reported [10].

Previously, dye-linked L-proline dehydrogenase (L-proDH) originally from hyperthermophilic archaeon (Therococcus profundus) has been expressed as a recombinant protein [11,12]. The enzyme showed high thermal stability, and a dye compound such as 2,6-dichlorophenolindophenol (DCPIP) could be used as an artificial electron acceptor for enzyme reaction. Our research aim is to develop an electrochemical L-proline sensing system based on this enzyme.

Design and preparation, especially immobilization of this enzyme is a crucial procedure in the construction of enzymatic biosensors. This is because enzymes should be stabilized and easily contacted with substrates, and the immobilization
procedure should be beneficial to electron transfer between enzyme and electrode surface by redox mediator [13,14]. As a novel method proposed by Decher and co-workers, layer-by-layer (LBL) adsorption method has been widely used in the assembly of nanoscale films composed of polyelectrolytes, such as proteins, DNA and, etc. It has been proved that LBL method is a promising way especially for the immobilization of biomolecules [15–19]. LBL method, which is based on electrostatic being forced between oppositely charged polyelectrolytes has been utilized on the immobilization of enzymes and construction of a biosensing system [20,21]. Various kinds of enzymes have been immobilized by LBL, and enzymes encapsulated in these integrated multilayer films showed superior chemical stability in long storage period and relative thermostability [22,23]. On the other hand, a biosensing system based on the alternated assembly of enzyme and polymerized mediator were fabricated, which made it possible to construct a reagentless sensor system [24–28]. In our previous work, LBL method has been used in the construction of a bienzyme biosensor [29].

In the present study, dye-linked L-proDH and polymerized mediator were fabricated on a gold electrode by LBL method. The electrochemical characteristics of the modified electrode and its application on the construction of l-proline biosensing system were investigated.

2. Experimental

2.1. Materials

Sodium borohydrde and 3-mercaptopropionic acid (3-MPA) were purchased from Nacalai Tesque Co. (Kyoto, Japan). Ferrocene carboxaldehyde, poly(allylamine) hydrochloride (molecular weight: 70,000) and triethylamine were obtained from Aldrich. The other reagents were of analytical grade.

2.2. Synthesis of polymerized mediator

The polymerized mediator was synthesized from poly(allylamine) and ferrocene carboxaldehyde according to the procedure reported by Hodak et al. [24]. Eight milligrams ferrocene carboxaldehyde was dissolved in 5 ml methanol and added dropwise within 1 h to 30 ml of methanolic solution containing 40 mg poly(allylamine) and 0.26 ml triethylamine. The mixture was stirred for 1 h at room temperature, then sodium borohydrde was carefully added in portions at 0 °C, and stirred continually for 90 min. Finally, the mixture was dried in vacuo condition and the residue was extracted with distilled water. The aqueous solution was purified by membrane dialysis against water. The polymer obtained was referred to as PAA-Fc and used for further experiments.

2.3. Purification of l-proDH

Recombinant dye-linked l-proDH originally from hyperthermophilic archaeon was prepared and purified according to previous procedures [11]. The activity of the enzyme was routinely determined spectrophotometrically by measuring the reduction rate of DCPIP at 595 nm according to the previous procedures described [11,12]. The reaction mixture was composed of 200 mM Tris–HCl buffer (pH 7.5), 0.1 mM DCPIP, 100 mM l-proline and enzyme solution with a total volume of 1.0 ml. One unit of activity was defined as the amount of enzyme that reduced 1 μmol of DCPIP per min at 50 °C. The molar absorption coefficient of $2.15 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ was used for calculating the reduction of DCPIP. The activity of the purified enzyme was measured at 6.0 units ml$^{-1}$ (with 3.0 mg protein).

2.4. Assembly of multilayer films

The gold electrode was finely polished by 1 μm diamond and 0.05 μm aluminum slurry and scanned over the potential range from +0.0 to +1.7 V vs. Ag/AgCl in 0.05 mM H$_2$SO$_4$ solution for about 30 min until the same cyclic voltammogram was obtained. The cleaned electrode was immersed in 10 mM 3-MPA ethanolic solution for 12 h, followed by rinsing with ethanol and water. A negative charged surface was achieved in this way.

Multilayer assembly was fabricated on 3-MPA modified electrode by alternative adsorption in PAA-Fc and l-proDH solution. The electrode was firstly immersed in 2 mg ml$^{-1}$ PAA-Fc solution for 20 min. Then it was rinsed with Mill-Q water and immersed in 3 mg-protein ml$^{-1}$ l-proDH (6.0 units ml$^{-1}$) in 10 mM phosphate buffer (pH 7.2) for another 20 min to immobilize the enzyme. A multilayer film could be obtained by repeating the above steps.

2.5. Electrochemical experiments

Electrochemical measurements were carried out on Hokuto Denko potentiostat HA-502 and function generator (HB-104) with an X–Y recorder, Model U-335 from Pantos Co. (Japan). A typical three electrode system was used, with Pt wire as the counter electrode, Ag/AgCl as the reference electrode and modified Au electrode as the working electrode. In all electrochemical experiments, 10 mM phosphate solution (pH 7.2) was used as the buffer, and the buffer solution was bubbling by using high purity nitrogen for more than 5 min before measurements to eliminate the influence of oxygen. A potential of +0.45 V vs. Ag/AgCl was set in amperometric experiments.

3. Results and discussion

The general properties of this thermostable dye-linked l-proDH were thoroughly characterized by Sakuraba et al. [11,12]. The enzyme (molecular weight 160 kDa) consists of four subunits ($\alpha\beta\gamma\delta$), and $\beta$ subunit catalyzes the dye-linked l-proline dehydrogenase reaction. As a dye-linked enzyme, l-proDH can use some organic compounds (such as DCPIP) as electron acceptors. For the construction of l-proline biosensing system, it is important to find a suitable mediator for l-proDH.
Because ferrocene and its derivatives are the most common mediators for various kinds of oxidoreductase, ferrocene carboxylic acid was used for preliminary experiments. Both L-proDH and ferrocene carboxylic acid were free in the buffer, and cyclic voltammograms were measured in the absence and presence of L-proline. Only specific redox peaks of ferrocene carboxylic acid were exhibited in the absence of L-proline. But after the addition of L-proline, the anodic peak increased dramatically and cathodic peak nearly disappeared. This confirmed the mediated reaction happened and ferrocene was an efficient mediator for this enzyme.

In order to develop reagentless biosensing system, a polymerized mediator (PAA-Fc) containing ferrocene groups as redox species was synthesized. Total iron content in the PAA polymer was estimated to be approximately 20% spectrophotometrically at 440 nm using a calibration curve for ferrocene carboxylic acid [24]. Both PAA-Fc and L-proDH were immobilized on Au electrode by LBL method. The reaction scheme is shown in Fig. 1.

### 3.1. Formation of PAA-Fc/L-proDH multilayer film on Au electrode and electrochemical behavior of the modified electrode

The adhesion layer was obtained after adsorption of 3-MPA (pK, 5.2) on Au electrode via its thiol end groups. At the buffer (pH 7.2), the electrode carries negative charges due to partially deprotonation of the carboxyl acid groups [30]. Because PAA-Fc carries positive charges and L-proDH carries negative charges under present experimental conditions, the multilayer film could be formed by an electrostatic force between oppositely charged polyelectrolyte and enzyme (as shown in Fig. 2). Characteristics of the electrode modified with PAA-Fc/L-proDH multilayer film were investigated by cyclic voltammetry. Fig. 3 shows the cyclic voltammograms of Au electrode modified with one to six PAA-Fc/L-proDH bilayers. A pair of oxidation–reduction peaks were obviously apparent (with midpoint at +0.37 V vs. Ag/AgCl). Both anodic and cathodic peaks increased with the number of PAA-Fc/L-proDH bilayers, which suggested that a multilayer structure had formed on the electrode surface. The amount of ferrocene groups in the multilayer film could be estimated by integrating the area of oxidation peaks in Fig. 3. The charge density of electrochemical active ferrocene was shown in the insert figure. It was observed that the amount of ferrocene groups increased with the bilayers, from one to five, but seemed to be saturated for the sixth bilayer. In the previous paper, QCM was used for measuring and analyzing the amount of enzyme and polymer, and a linear increase of adsorption amount was found for assembly more than 10 bilayers by LBL method [19]. So, it could be evaluated that nearly same amount of Fc in each
mediator layer. For multilayer assembly, Fc moieties on the inner layers are electrochemically active, but those moieties on the outer layers may not be active because electron hopping is not sufficient.

The electrochemical characteristic of the multilayer film was further studied under different scan rates. Cyclic voltammetric experiments of Au electrode modified with three and six PAA-Fc/L-proDH bilayers were carried out at different scan rates (from 40 to 200 mV s\(^{-1}\)), and the influence of the scan rate on anodic current peaks is shown in Fig. 4. The anodic peak currents increased linearly with the scan rate for Au electrode modified with three PAA-Fc/L-proDH bilayers, indicating a diffusion-free electron transfer between ferrocene groups and electrode. On the other hand, for Au electrode modified with six PAA-Fc/L-proDH bilayer film, the anodic peak currents did not depend linearly but were proportional to the square root of the scan rate. As illustrated by Liu et al. [31], a charge diffusion contributes more or less to electron transfer for the thicker films fabricated by LBL method. This might be the reason for the phenomena observed in these experiments.

3.2. Electrocatalytic oxidation of l-proline on electrode modified with PAA-Fc/l-proDH multilayer film

The response of the multilayer film modified electrode to l-proline was characterized by cyclic voltammetry. Cyclic voltammograms of Au electrode modified with four PAA-Fc/
L-proDHred + PAA-Fc → L-proDHox + PAA-Fc

PAA-Fc → e⁻ + PAA-Fc⁺

L-proline diffused to the surface of the multilayer film modified electrode, and was oxidized to pyrroline-5-carboxylate by immobilized L-proDHox. The L-proDHred produced in the enzymatic reaction, reduced the mediator PAA-Fc to PAA-Fc. Finally, the PAA-Fc produced was then electrochemically oxidized and gave anodic current response. By this way, the communication between immobilized enzyme and electrode was achieved which is essential for the construction of biosensing system, and the immobilized L-proDH was 'wired' by PAA-Fc.

3.3. Steady-state current response of the modified electrode to l-proline

The typical steady-state amperometric response of gold electrode modified with four PAA-Fc/l-proDH bilayers is shown in Fig. 6. The applied anodic potential was set at +0.45 V vs. Ag/AgCl. It was found clearly that anodic current increased with the injection of l-proline solution.

The influence of bilayer number on amperometric response was further investigated and shown in Fig. 7. The current response to substrate increased with the bilayer number two to six. It was attributed to the more immobilized enzyme and mediator with the layer number increasing. But the current response obtained was relatively low which might be due to the low activity of the enzyme solution used in the fabrication of the multilayer film. Now efforts are being made to prepare a more concentrated enzyme solution with high activity.

3.4. Long-term stability of the multilayer film

The stability of the l-proline sensor was also investigated by measuring its current response to l-proline during long-term storage. Au electrode was modified with four PAA-Fc/l-proDH bilayers, and its response to 8 mM l-proline was measured. The modified electrode was stored in the buffer under 4 °C when not in use. The relative response after long-term storage is shown in Fig. 8. No decrease in response to l-proline was observed for the first 12 days, which suggested that the enzyme kept its activity after immobilization by LBL method, and the multilayer film was stable. The response decreased continuously after 12 days. It seemed to be due to the deactivation of l-proDH, desorption of PAA-Fc, or the enzyme from the multilayer film. About 40% of the relative response was retained after 30 days storage.

4. Conclusion

In the present work, LBL method has been utilized in the construction of l-proline sensor based on multilayer assembly of polymerized mediator (PAA-Fc) and dye-linked l-proDH on Au electrode. Cyclic voltammetry showed clearly redox peaks of ferrocene, and both cyclic voltammetry and amperometry experiments proved that catalyzed electrochemical oxidation of l-proline was achieved on a modified electrode, which
confirms that electrochemical communication has been achieved between immobilized enzyme and electrode by the polymerized mediator in the multilayer film. The current response to L-proline increased with PAA-Fc/L-proDH bilayer number, and the L-proline sensor with multilayer film showed relatively long-term stability.

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