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Development of a D-amino acids electrochemical sensor based on immobilization of thermostable D-Proline dehydrogenase within agar gel membrane

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ABSTRACT

A novel biosensor for determination of D-amino acids (DAAs) in biological samples by using an electrode based on immobilization of a thermostable D-Proline dehydrogenase (D-Pro DH) within an agar gel membrane was developed. The electrode was simply prepared by spin-coating the agar solution with the D-Pro DH on a glassy carbon (GC) electrode.

An electrocatalytic oxidation current of 2,6-dichloroindophenol (DCIP) was observed at −100 mV vs. Ag/AgCl with the addition of 5 and 20 mmol L^{-1} D-proline. The current response and its relative standard deviation were 0.15 μA and 7.6% ($n = 3$), respectively, when it was measured in a pH 8.0 phosphate buffer solution containing 10 mmol L^{-1} D-proline and 0.5 mmol L^{-1} DCIP at 50 °C. The current response of D-proline increased with increase of the temperature of the sample solution up to 70 °C. The electrocatalytic response at the D-Pro DH/agar immobilized electrode subsequently maintained for 80 days. Finally, the D-Pro DH/agar immobilized electrode was applied to determination of DAAs in a human urine sample. The determined value of DAAs in the human urine and its R.S.D. were 1.39 ± 0.12 mmol L^{-1} and 8.9% ($n = 3$), respectively.

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1. Introduction

Recently, D-amino acids (DAAs) have been researched in various scientific fields [1–3]. For example, it has been known that D-serine has a function to adjust glutamatergic neurotransmission in brain [4,5]. Furthermore, D-asparatic acid, which is usually found in an internal secretion of rats, may control production of hormones [6–8]. D-Proline has been also found in human saliva [9], gastric juice [10], mouse urine [11], brain [12], beer [13], and maple syrup [14]. In addition, an accumulation to stomach cancer [10] and neural toxicity of D-proline [15,16] was reported. However, biological functions of DAAs including D-proline to human body have been rarely cleared. Therefore, reliable determination methods for DAAs are necessary for clarifying their biological and physiological roles.

Several analytical methods such as high-performance liquid chromatography (HPLC), gas chromatography (GC) and amino acid analyzer have been utilized for the determination of DAAs in biological and food samples because of their sensitivity and selectivity [17]. However, these instruments are

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generally expensive and large. Thus, enzyme-based amperometric biosensors are useful as an alternative method because they have many advantages of high selectivity for analytes and compact mobility of the analytical system. The enzyme-based amperometric biosensors have been applied to determination of many compounds in biological and food fields [18–20]. A few applications of enzyme-based amperometric biosensors with which mesophile D-amino acid oxidase (m-DAAOx) and L-amino acid oxidase (m-LAAOx) were commonly utilized as a sensor element [21–26] have been reported. For examples, L-leucine in milk, fruits juice and urine was determined by m-LAAOx immobilized screen-printed electrode [24]. Furthermore, D-alanine in drinks and L-phenylalanine in human urine could be detected by a screen-printed electrode immobilized with m-DAAOx/Prussian Blue and m-LAAOx/osmium complex immobilized electrode, respectively [25,26]. These conventional enzyme sensors for determination of amino acids still had a lack of long-term stability caused from deactivation of the immobilized enzymes. The long-term stabilities of the m-DAAOx immobilized sensors were less than 1 month [21–23,25,26]. Thus, we focused on improvement of long-term stability of amino acids sensor.

We developed an electrochemical DAAs sensor in which a thermostable D-Proline dehydrogenase (D-Pro DH) was used as a DAAs sensor element. Thermostable enzymes usually have high stability under extreme environments such as high temperature, highly acidic and alkaline medium [27,28]. It is expected that the use of thermostable enzyme for electrochemical biosensors contributes to improve the sensor stability, which leads to expand possibilities of developing electrode preparation and its usage situation. Thermostable glucose-6-phosphate dehydrogenase and asparaginase have been applied to enzyme-based biosensors [29,30].

The D-Pro DH was firstly purified from *Pyrobaculum islandicum* and characterized by Satomura et al. [31]. The D-Pro DH showed high activity to DAAs and especially D-proline. Thermostable enzymes with high selectivity and activity to DAAs have been rarely found. Furthermore, there has been no application of thermostable enzymes to DAAs biosensors.

Taking into account of the development of stable enzyme sensor, immobilization of the enzyme on the surface of the electrode is also one of the most important factors in the electrode preparation and its use. Generally, the performance mainly depends on the nature and immobilized conditions of enzymes. In this work, we proposed to use an agar as an immobilization matrix of D-Pro DH. Agar, which is a thermosensitive polymer composed of agarose, shows a thermosensitive sol–gel transformation which forms a random coil structure above 80 °C and a helix structure with the three-dimensional networks less than 80 °C [32,33]. The thermosensitive sol–gel transformation and the high physical stability with agar are very useful to immobilize thermostable enzymes. It is expected that the enzyme immobilization by spin-coating agar gel solution is simple and rapid. The application of agar to an immobilization matrix of thermostable enzymes has not been reported yet.

In this report, our approach to the development of highly stable DAAs enzyme-based amperometric sensor was to use the thermostable D-Pro DH as a sensor element within agar gel as the immobilization matrix. The D-Pro DH/agar immobilized electrode was firstly characterized by electrochemical analysis. The preparation of the D-Pro DH/agar immobilized electrode and the analytical conditions of D-proline were also optimized. Finally, we tried to apply the determination of DAAs in a urine sample by the D-Pro DH/agar immobilized electrode.

2. Experimental

2.1. Reagents

Phosphoric acid, potassium chloride, 2,6-dichloroindophenol (DCIP), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate 12-water, agar, D-glutamate acid, D-leucine, D-valine and, D-alanine were of analytical grade and purchased from Kanto Chemical Co. (Tokyo, Japan). D-Proline, D-histidine, D-aspartate acid, D-serine, D-isoleucine and L-proline were of analytical grade and purchased from Wako Pure Chemical Industries (Osaka, Japan). A frozen human urine single donors unit (male) was obtained from BIOPREDIC (France).

2.2. D-Proline dehydrogenase (D-Pro DH)

The D-Pro DH, which originates from hyperthermophilic archaeon *Pyrobaculum islandicum*, is a membrane-associated thermostable enzyme. It was obtained by using the method reported by Satomura et al. [31] and stored in a solution containing 50% glycerol and 1% Triton at 4 °C. The molecular weight and the coenzyme of the D-Pro DH are 42 kDa and flavin adenine dinucleotide (FAD), respectively. The activity of the D-Pro DH was 6.4 units. It has been already known that DCIP was effective as an electron mediator for D-Pro DH by spectrophotometric analysis [31]. Fig. 1 shows a schematic diagram of electron transferring reactions of D-Pro DH and DCIP on the surface of the electrode.

2.3. Electrochemical measurement

All electroanalytical experiments were performed by using a potentiostat BAS-100B/W (BAS Inc., USA) equipped with a three-electrode system. The working electrode was the D-Pro DH/agar immobilized glassy carbon (GC) electrode. The counter-electrode was a platinum wire and an Ag/AgCl electrode (saturated KCl) served as reference. Electrochemical experiments were performed by a batch system. Cyclic voltammograms were normally recorded at scan rate of 1 mV s^{-1}. A timebase technique in which the applied potential

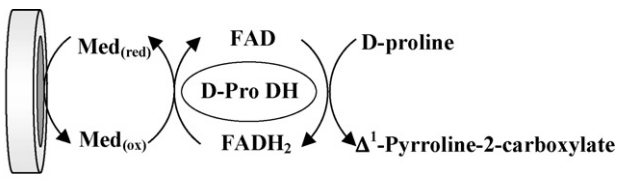

Fig. 1 – Schematic diagram of electron transferring reactions on a D-Pro DH/agar immobilized electrode.

held a constant at 300 mV vs. Ag/AgCl was used for the determination of D-proline. DCIP was dissolved in the 0.067 mol L^{-1} phosphate buffer solution (PBS) at pH 8.0. Before the electrochemical measurement, nitrogen gas was passed into the PBS. The measurement temperature was ordinary set at 50 °C by temperature-controlled bath (Type TR-2A, As One Corporation, Osaka, Japan). The temperature of analytical solution was measured by a thermosensor with the temperature-controlled bath. In an experiment for the determination of DAAs in human urine, the human urine (1 mL) was taken in a glass vial and electrolyzed with Pt mesh electrode at 1300 mV vs. Ag/AgCl for 1 h. Subsequently, the sample was analyzed by the three-electrode system.

2.4. *Preparation of the enzyme electrode*

First, a GC electrode was sequentially polished with 6 μm and 1 μm of diamond paste and 0.05 μm alumina powder. The electrode was then rinsed by pure water. The electrode was set on an electrode-rotating machine for spin-coating the agar gel with D-Pro DH. Fifteen microliters of the solution containing 1.5% (w/v) agar and 6.4 units D-Pro DH heated at 80 °C was dropped onto the surface of the electrode rotating at 4000 rpm. The electrode was cooled at room temperature for 15 min and used for the electroanalytical measurement after rinsing its surface with pure water.

UV–vis spectrophotometer (Jasco Model V-570, Tokyo, Japan) was used to check the amount of D-Pro DH in agar gel immobilized on the surface of the electrode. In this experiment, the D-Pro DH/agar immobilized electrode was soaked in 1 mL of the pH 8.0 PBS at 80 °C to dissolve the agar gel on the surface of the electrode. Subsequently, the amount of D-Pro DH in the agar dissolved solution was determined by the UV–vis spectrophotometer at 250 nm [34].

3. Results and discussion

3.1. *Preparation of the D-Pro DH/agar immobilized electrode*

The D-Pro DH/agar immobilized electrode was characterized by cyclic voltammetry. The cyclic voltammogram was measured in pH 8.0 PBS containing 0.5 mmol L^{-1} DCIP and 0.1 mol L^{-1} KCl. The voltammograms were shown in Fig. 2. The electrocatalytic oxidation currents of DCIP were observed at −100 mV vs. Ag|AgCl and increased with the addition of 5 and 20 mmol L^{-1} D-proline. On the other hand, the electrocatalytic oxidation current of DCIP was not observed with a bare GC electrode and an agar immobilized electrode. These results indicate that the activity of D-Pro DH in the agar gel membrane on the surface of the electrode was maintained during its preparation and the measurement.

The electron transferring process occurring on the surface of electrode was summarized as follows:

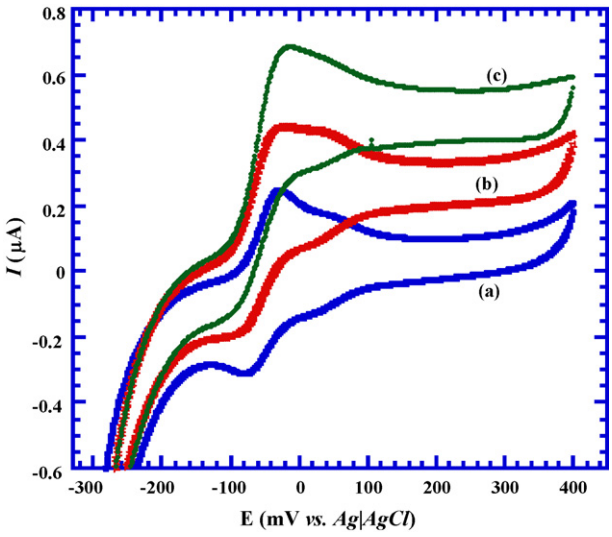

Fig. 2 – Cyclic voltammograms of electrocatalytic oxidation current of DCIP with a D-Pro DH/agar immobilized electrode. The cyclic voltammograms were observed in 10 mL pH 8.0 PBS containing 0.5 mmol L^{-1} DCIP, 0.1 mol L^{-1} KCl, without (a), with (b) 5 and (c) 20 mmol L^{-1} D-proline at 50 °C. The scan rate was 1 mV s^{-1} in the all measurements.

D-Pro DH-FAD + D-proline

→ D-Pro DH-$FADH_2$ + Pyrroline-2-carboxylate (1)

D-Pro DH-$FADH_2$ + $2Med_{(Ox)}$

→ D-Pro DH-FAD + $2Med_{(Red)}$ + $2H^+$ (2)

$2Med_{(Red)} \rightarrow 2Med_{(Ox)} + 2e^-$ (3)

As can be seen in Fig. 2, the voltammogram of the catalytic oxidation current in the addition of D-proline at the D-Pro DH/agar immobilized electrode was not sigmoidal shape. The non-sigmoidal shape of the voltammogram may be due to two causes which were low concentration of the immobilized D-Pro DH on the surface of the electrode or slow diffusion rate of the electron mediator and lack of permeability of D-proline. Thus, the reason for the non-sigmoidal voltammogram was tried to be identified by the next experiment, in which the analytical solution was mechanically stirred during the measurement of the cyclic voltammetry to supply D-proline and DCIP to the surface of the electrode forcibly. As a result, the voltammogram under the stirring condition was similar to that under the non-stirring. It was found from this result that the non-sigmoidal voltammogram was scarcely related to the permeability of the substrate and the diffusion process of the electron mediator. Consequently, the non-sigmoidal voltammogram was originated from the electron transferring process controlled by the enzyme kinetics.

Agar concentration affects gel strength which may strongly correlate the amount and the stability of immobilized enzymes in the gel. Then, we investigated the effects of the agar concentration on the current response and the amount of immobilization enzyme. Fig. 3 shows the effects of the agar concentrations between 0.25 and 2.0% (w/v) on the oxidation current observed in 10 mmol L^{-1} D-proline solution (ΔI) and on

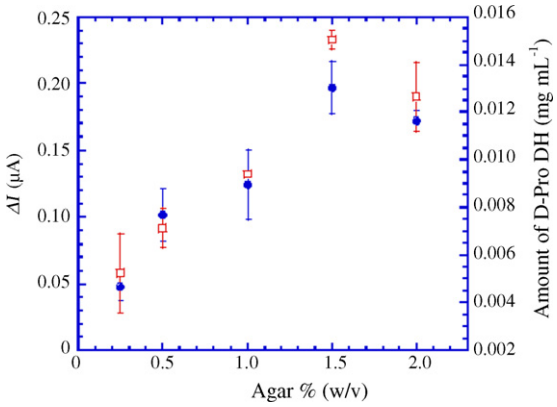

Fig. 3 – Relationship between agar concentration in the preparation of a D-Pro DH/agar immobilized electrode and the current response of D-proline (Y-axis) and the amount of D-Pro DH in the agar gel membrane (Z-axis). ΔI; the current response was observed in 10 mL pH 8.0 PBS containing 10 mmol L^{-1} D-proline, 0.5 mmol L^{-1} DCIP and 0.1 mol L^{-1} KCl at 50 °C. The marks (●) and (□) indicate the current response of D-proline and the amount of D-Pro DH, respectively.

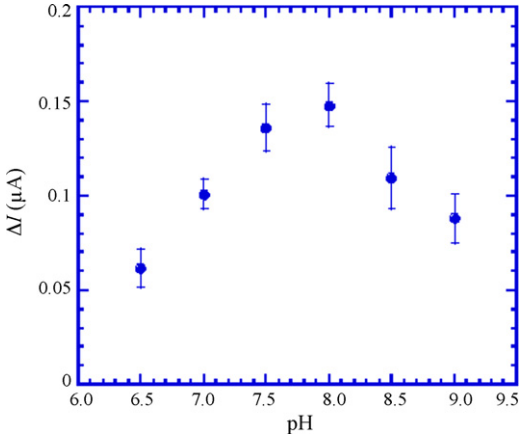

Fig. 4 – Dependence of the current response of D-proline with a D-Pro DH/agar immobilized electrode on the pH of the analytical solution. ΔI; the current response was observed in 10 mL buffer solution containing 10 mmol L^{-1} D-proline, 0.5 mmol L^{-1} DCIP and 0.1 mol L^{-1} KCl at 50 °C.

the amount of D-Pro DH in the agar gel membrane. As can be seen in Fig. 3, both the current response and the amount of D-Pro DH were maximized at 1.5% (w/v) of the agar concentration. From this result, the current response of D-proline was strongly correlated with the amount of immobilized enzyme in the agar gel membrane. In the low agar concentration less than 1.5% (w/v), the low current response might arise from the low amount of the immobilized enzyme due to desorption of the enzymes from the agar gel membrane. On the other hand, the agar at the concentration over the 2.0% (w/v) was incompletely dissolved with heating up 80 °C. An inhibition of permeability of D-proline and decrease of the diffusion of DCIP to the surface of the electrode may occur in the agar condition, since the agar membrane was formed non-uniformly and very thickly. In this work, the optimum agar concentration was chosen 1.5% (w/v).

3.2. *Characterization of the D-Pro DH/agar immobilized electrode*

We optimized analytical conditions of the D-Pro DH/agar immobilized electrode. Fig. 4 shows the current response of 10 mmol L^{-1} D-proline at various pH conditions between 6.5 and 9.0. The maximum current response and its relative standard deviation were observed at pH 8.0 and less than 10%. While, the R.S.D.s at the other pH points were *ca.* 15%. The agar gel used as an immobilization matrix of D-Pro DH is generally stable in the pH range from 4 to 10 [35]. Therefore, it was suggested that the pH behavior of the current response reflected the pH dependence of the activity of D-Pro DH, with which the pH of the highest activity was 8.0 [31]. Consequently, the pH 8.0 was used as the optimum pH condition in further measurements.

Fig. 5 shows the plots of the current response of 10 mmol L^{-1} D-proline at various temperatures of the analytical solution. As can be seen in Fig. 5, the current response increased with increase of the measurement temperature up to 70 °C. The R.S.D.s were less than 10% at all measurement temperatures. The temperature dependency was agreed with a thermal trend of the activity of D-Pro DH itself [31]. This result also shows that the immobilized thermostable D-Pro DH on the surface of the electrode stably existed in the agar gel membrane under the high temperature. While, the conventional m-DAAOx immobilized electrode faced a limitation of the thermal stability up to only 40 °C because of the deactivation of the immobilized enzyme [21].

In the optimum analytical condition, the linear regression of the D-proline concentration was observed from 0.5 to 5.0 mmol L^{-1} (correlation coefficient = 0.997) and the Michaelis–Menten constant was 7.9 mM.

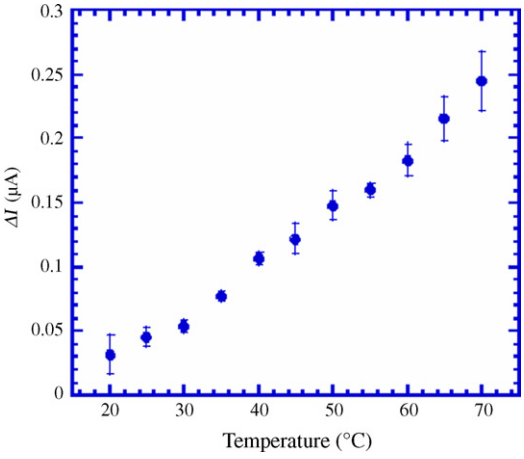

Fig. 5 – Dependence of the current response of D-proline with a D-Pro DH/agar immobilized electrode on the temperature of analytical solution. ΔI; the current response was observed in 10 mL pH 8.0 PBS containing 10 mmol L^{-1} D-proline, 0.5 mmol L^{-1} DCIP and 0.1 mol L^{-1} KCl at various temperature.

Table 1 – Substrate selectivity to amino acids with the D-Pro DH/agar immobilized electrode

Substrate	Selectivity (%)[a]
D-Proline	100
D-Isoleucine	46
D-Histidine	29
D-Leucine	19
D-Valine	18
D-Alanine	9
D-Aspartate	7
D-Serine	0
D-Glutamate	0
L-Proline	0

[a] The substrate selectivity was defined as the percentage of the current response observed in 10 mmol L^{-1} D-proline to that in the same concentration of the amino acid at the D-Pro DH/agar immobilized electrode.

3.3. *Selectivity of D-Pro DH to other amino acids*

The selectivity of amino acids observed at the D-Pro DH/agar immobilized electrode was examined. The result is listed in Table 1. The percentage was obtained by measuring the current response at 10 mmol L^{-1} D-proline solution versus that at the same concentration of the other amino acids solution. The D-Pro DH/agar immobilized electrode was reacted to D-isoleucine (46%) and several DAAs, while it has high selectivity to D-serine, D-glutamate and other L-amino acids including L-proline. These results indicate that the D-Pro DH electrode reacts to only DAAs. The selectivity with D-Pro DH itself to these amino acids was reflected in the selectivity of the D-Pro DH/agar immobilized electrode [31].

3.4. *Reproducibility and long-term stability of the D-Pro DH/agar immobilized electrode*

Reproducibility and long-term stability of current response are also very important factors to develop and use a biosensor. In the experiment, the reproducibility and the long-term stability were assessed in pH 8.0 PBS containing 10 mmol L^{-1} D-proline at 50 °C. As a result of the reproducibility experiment including the electrode preparation and the measurement of D-proline at three times, the current response and its relative standard deviation were 0.15 μA and 7.6%, respectively. This result indicates that a uniform membrane of the agar gel was formed on the surface of the electrode without loss of the activity of D-Pro DH. The reproducibility of this electrode was similar to those of the conventional enzyme sensors, which were constructed by screen-printed method [24], graphite–Teflon [36] and sol–gel method [37].

The long-term stability of the D-Pro DH/agar immobilized electrode was evaluated by the current ratio (I/I_0). The I_0 was defined as the initial current measured in the first day of the preparation of the electrode. The D-Pro DH/agar immobilized electrode was stored at room temperature under dry and ambient conditions, when it was not used. The long-term stability of the D-Pro DH/agar immobilized electrode was tested by measuring on every 7 days up to day 28, day 45 and day 80 from the first day of the preparation of the electrode. As can be seen in Fig. 6, the current ratio retained over 90% for 80 days. The conventional m-DAAOx immobilized carbon paste and Pt micro-electrode showed the long-term stability for 30 days [21,26]. Furthermore, the other conventional m-DAAOx immobilized electrodes based on electro-polymerization [22,23] and screen-printed method [25] had 1 or 2 weeks of the long-term stabilities. Consequently, the high physical robustness of the agar gel and the high activity with the thermostable D-Pro DH were contributed to the high long-term stability of the D-Pro DH/agar immobilized electrode.

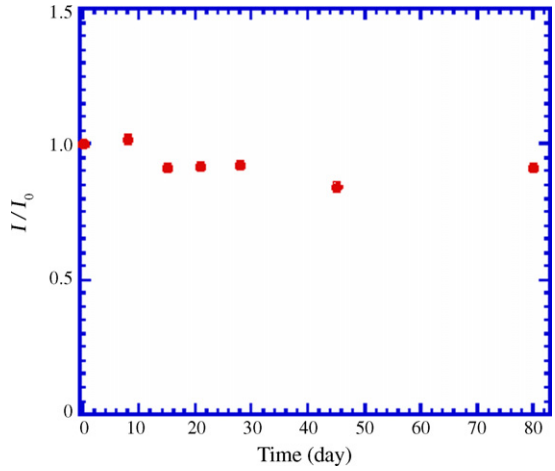

Fig. 6 – Long-term stability of the current response with a D-Pro DH/agar immobilized electrode. I_0; the initial current response was measured in 10 mL pH 8.0 PBS containing 0.5 mmol L^{-1} DCIP, 0.1 mol L^{-1} KCl and 10 mmol L^{-1} D-proline at first day to prepare the electrode. I; the current response was measured at the day. The enzyme electrode was stored at room temperature under a dry condition.

3.5. *Determination of DAAs in human urine*

In this study, the D-Pro DH/agar immobilized electrode was applied to determination of DAAs in a human urine sample. The D-Pro DH/agar immobilized electrode reacts several DAAs such as D-isoleucine, D-histidine and D-leucine as mentioned in Section 3.3. Therefore, it is thought that the determination value deals with the total concentration of DAAs in the human urine. The uric acid in human urine often interferes in the determination of analytes in a biological sample by amperometric analysis [38]. Thus, the electrolysis of the uric acid at about 1300 mV vs. Ag/AgCl for an hour was tested to urine samples to remove it prior to the determination of DAAs. The oxidation peak of the uric acid was completely disappeared after the electrolysis. The determination value of DAAs in the human urine and its R.S.D. were 1.39 ± 0.12 mmol L^{-1} and 8.9% ($n = 3$), respectively. However, the reliability of the value has not been certified, because there is very little consensus of normal values of DAAs including D-proline in biological samples. For example, there were a few values for only D-proline, D-alanine, D-leucine, D-serine, D-phenylalanine and D-aspartic acid and no information of the normal values of other DAAs such as D-isoleucine and D-histidine in a review for values of DAAs in biological samples [17]. Furthermore, the concentration of

D-alanine and D-proline in mammals has a significant variation among a day according to the reports [9,39]. In addition, standard reference materials with certified values for DAAs have not been issued. Therefore, cross-checking for determination of DAAs in multiple number samples or development of standard reference materials for DAAs should be a promised further research to establish consensus values of DAAs.

4. Conclusion

We developed a thermostable D-Pro DH/agar immobilized electrode. The sensor preparation based on a spin-coating method of the agar solution containing a thermostable D-Pro DH was very simple and rapid. The current response of electrocatalytic oxidation of D-proline could be maintained in the analytical solution temperature up to 70 °C. Furthermore, the D-Pro DH/agar immobilized electrode was stable up to 80 days. The long-term stability of the D-Pro DH/agar immobilized electrode was superior to those of the other conventional DAAs biosensors. The determination value of DAAs in the human urine was obtained by the D-Pro DH/agar immobilized electrode. The D-Pro DH/agar immobilized electrode can be utilized as the portable screening devices for DAAs in biological and food sample.

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