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BIOTECHNOLOGY PROGRESS

RESEARCH ARTICLE

Process Sensing and Control

Electrochemical characteristics of a hyperthermophilic enzyme in microdroplets stirred and heated by surface acoustic waves

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Abstract

Micro total analysis system (μTAS) is expected to be applied in various fields. In particular, since electrochemical measurement is inexpensive and easy, electrochemical measurement can be integrated with a microchannel. However, electrochemical detection sensitivity in a microchannel is lowered because the diffusion of the detection target is limited. In an ordinary electrochemical detection system, using a stirrer in a beaker can overcome limited diffusion. We previously proposed a new detection system that combines a microliquid solution agitation technology using surface acoustic waves (SAWs) with the μTAS. The SAWs function as microstirrers, thus making electrochemical detection possible by overcoming limited diffusion of the sample. However, when the solution is stirred by the SAWs, the temperature of the solution increases to 70°C due to vibrational energy. This leads to enzyme inactivation and impaired electrochemical response. Therefore, in this study, we used a hyperthermophile-derived enzyme. Temperature and electrochemical characteristics of the detection system using SAWs and a multi-copper oxidase (MCO) derived from the hyperthermophilic archaea *Pyrobaculum aerophilum* were studied. Laccase, which is an MCO derived from the thermophilic fungus *Trametes versicolor*, was also studied. We also characterized the enzyme-electrochemical reaction using SAWs by comparing the magnitude of the reduction current obtained using the two enzymes with different heat resistances. We observed an increase in the electrochemical response with the SAWs, without impaired enzyme activity. Thus, the use of a thermostable enzyme is suitable for the development of a biosensor that uses SAWs for agitation.

KEYWORDS

amperometry, electrochemical detector, micro-stirrer, surface acoustic waves, thermostable enzyme

1 | INTRODUCTION

In recent years, with growing interest in health, food safety, environmental problems, etc., there is a need for a sensor that can easily measure pollutants and contaminants on the spot.[1] For example, in the field of medicine, point-of-care testing (POCT), which can be performed by patients themselves, has gained popularity.[2-5]

Furthermore, to develop a practical analysis system, micro total analysis system (μTAS), which is a detection technology that uses a microchannel manufactured by microfabrication technology, has gained attention.[6] The μTAS is portable because of its miniature size, and it requires only a small amount of sample for detection.[7,8]

In an ordinary electrochemical detection system, the sensor electrode is placed in a few milliliters of sample, and it detects the given substance with high sensitivity. The sample is mixed using a stirrer to facilitate diffusion, so that the solution is homogenous and accurate detection is possible. On the other hand, in μTAS, a nano-/microliter of fluid is used, which makes it difficult to stir the sample using a stirrer. In this case, diffusion of the molecules to be detected at the electrode surface depends on natural diffusion, decreasing current and, thereby, sensor sensitivity.

To solve this problem, we focused on a solution agitation technology using surface acoustic waves (SAWs) as an actuator.[9-11] A SAW is a wave in which the energy is concentrated and propagated on the surface of an elastic body. SAWs are generated by applying a high-frequency voltage to a comb electrode (IDT: interdigital transducer) manufactured on a substrate of a piezoelectric material. Previously, Saiki et al. fabricated an IDT with a simple structure of 5 mm^2 (50 μm in line and space and 4,000 μm in aperture length) on an artificial ceramic lithium niobate ($LiNbO_3$) substrate and demonstrated the transportation and stirring of the small amount of liquid placed on the propagation surface by the SAWs.[12,13] In our previous study, we proposed a highly sensitive electrochemical system by combining solution agitation using SAW and the electrochemical detection system μTAS. We observed that by stirring the solution using SAWs, the redox molecule was easily diffused into the electrode surface, and the measured current value significantly increased. By using this device, we solved the problem of low sensitivity of the electrochemical system due to microanalysis.[14]

Our previous study has shown that the stirring effect of SAWs promotes the electrochemical reaction characteristics of a low-molecular-weight metal complex, ferrocene.[14] However, the detection of the redox reaction between an enzyme and a substrate is hampered by the slow diffusion rate of the high-molecular-weight enzyme. Therefore, increasing the diffusion rate and promoting the enzyme reaction by SAW would be a very significant improvement.

We observed that the temperature of the solution increased depending on the power supplied to the IDT after 170 s of SAW generation, reaching up to 71.6°C.[12] The increase in temperature of the solution could be caused by the vibrational energy of the SAWs, which was converted to heat on the substrate. Further, the temperature change could also depend on the supplied power because the energy and amplitude of the generated SAWs increase as the supplied power increases.

This increase in the temperature is a challenge in developing an enzyme biosensor utilizing the stirring effect of the SAWs. Enzymes in living organisms, including humans, usually show maximum activity around 40°C, which is near body temperature; these enzymes denature at temperatures of 50 to 60°C. Thus, the inactivation of the enzymes due to heating by SAWs could impair sensor performance. To solve this problem, in this study, we focused on a thermostable enzyme obtained from a hyperthermophile living at temperatures above 70°C. Hyperthermophilic archaeon are microorganisms that grow in high-temperature environments, such as hot springs. Because of this, these types of enzymes can maintain activity for prolonged times without the necessity for deactivation or denaturation. The enzymes from hyperthermophilic archaeon were able to utilize at temperatures from 50 to 90°C. We hypothesized that incorporating this thermostable enzyme into the SAW device would allow the construction of a highly sensitive biosensor that utilizes the increase in temperature to increase the enzyme reaction rate. We used McoP, a multi-copper oxidase (MCO) derived from the hyperthermophile *Pyrobaculum aerophilum*, as a model thermostable enzyme.[15] MCO has four copper atoms and can catalyze the reduction of four electrons from oxygen molecules; thus, it is often used as cathode for biobatteries.[16-18] Since McoP has an optimal temperature of 85°C, it retains its activity even under high-temperature conditions. We measured the catalytic reduction of oxygen by McoP electrochemically, while changing the power supplied to the IDT. In addition, the reduction of oxygen by laccase, which is an MCO derived from the thermophilic fungus *Trametes versicolor*, was also measured. Laccase retained its activity both after incubation at 40°C for 10 min and after incubation in the range of pH 4.5 for 10 min. We also characterized the enzyme-electrochemical reaction using SAWs by comparing the magnitude of the reduction current obtained using the two enzymes with different heat resistances.

2 | MATERIALS AND METHODS

2.1 | Materials

Acetic acid, glycine, hydrochloric acid (HCl), and sodium acetate were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was laccase from *Trametes versicolor* purchased from Sigma-Aldrich Co., LLC (St. Louis, MO). All chemicals were of analytical grade. Deionized water filtered through a Milli-Q water purification system (Millipore Co., Bedford, MA) was used for experiments in this study.

2.2 | Design and working mechanism

Figure 1 shows a schematic illustration of the electrochemical detector incorporated with the SAW micro-stirrer. The detector with the

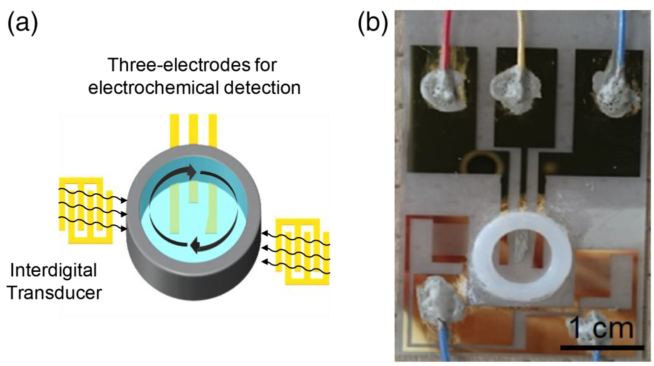

FIGURE 1 Schematic illustration of liquid flow with SAW stirring: (a) bird view of the device and (b) photograph of the fabricated electrochemical detector with SAW microstirrer

SAW micro-stirrer consists of two IDTs, a three-electrode system patterned on a piezoelectric substrate, and a polymer ring used as a wall of the liquid pool. When a high-frequency voltage is applied to the two IDTs, SAWs were generated. The SAWs propagated through the substrate surface and passed through the bottom of the polymer ring. When the SAWs reached the bottom part of the liquid pool, liquid in the pool became energized by the longitudinal pressure wave radiation. The two IDTs were placed on each side of the liquid pool, and their axes shifted along the central axis of the liquid pool. Thereby, a rotational flow of the liquid was produced efficiently within the polymer ring, which effectively increased the diffusion rate of the analyte. The three-electrode system for electrochemical measurement and the SAW micro-stirrer were fabricated using UV lithography and liftoff process on a piezoelectric substrate. Detailed fabrication methods and SAW drivers and conditions are described in the previous article.[14]

2.3 | Solution temperature measurement during SAW drive

An infrared thermography camera (Model. T400, FLIR, OR) was used to record the temperature of the center point of the solution in the O-ring at 210 s after SAW generation. A 60 μL of ultrapure water was dropped into the O-ring, and the power supplied to the IDT was varied in the range of 0.1‐1.0 W. The measurement parameters were: emissivity ε = 0.96, reflection temperature = 20°C, distance = 0.2 m, relative humidity: room humidity, ambient temperature: room temperature.

2.4 | Preparation of McoP

McoP was expressed in *E. coli* BL21-Codon-Plus (DE3)-RIPL, as previously described.[15] McoP was purified from a soluble extract to obtain high purity through a three-step process using heat treatment (80°C, 10 min), anion-exchange chromatography, and gel filtration chromatography. The molecular weight, purity, and enzyme activity of McoP were determined, as previously described.[15]

2.5 | Electrochemical measurement

Electrochemical measurement was performed by using a Model 800B Electrochemical Analyzer (BAS Inc, Tokyo, Japan). The three-electrode system consisted of Au working and counter electrodes and an Ag/AgCl reference electrode. All potentials were referred to the Ag/AgCl electrode, and a constant potential was applied to the working electrode vs. Ag/AgCl. Glycine-HCl buffer (45.6 μL; 100 mM, pH 3.0) and ABTS (2.4 μL; 20 mM) were dropped into the liquid pool, and the constant potential of 100 mV was applied to the working electrode. After 20 s, the SAWs were generated by applying a voltage to the interdigital transducer (IDT) on the substrate, and the solution in the O-ring was started stirring. After another 10 s, 10 μL of a 5.0 mg/mL enzyme (McoP or laccase) solution was dropped into the O-ring and current was measured until 230 s. By comparing the reduction current values 230 s after the start of the potential application, the reduction current of oxygen by the catalytic reaction of the enzyme was measured while stirring by the SAWs.

3 | RESULTS AND DISCUSSION

3.1 | Solution temperature change by SAW

The temperature of the central point of ultrapure water in the O-ring was recorded 210 s after SAW generation (at 20 s), while changing the power supplied to the IDT (Figure 2). The temperature increase occurred immediately after the SAW drive, and the temperature remained constant after 200 s. Thus, it is clear that the rate of temperature increase and the saturation temperature change depend on the magnitude of the supplied power. When the power supplied was 0.1, 0.5, and 1.0 W, the temperature of the solution increased to 23.3, 43.3, and 60.3°C, respectively, which is the temperature at which a non-thermostable enzyme gets inactivated. This was likely because the solution was heated by the vibrational energy of the SAWs.

3.2 | Change in enzyme activity due to temperature increase by SAW agitation

The enzyme activities of laccase and McoP were measured under each temperature condition generated by the SAW drive obtained from Figure 2. The relative activity change was compared with the highest activity being set to 100% (Figure 3). The activity of the thermostable enzyme McoP became higher as the temperature increased. On the other hand, it was revealed that laccase activity decreased to 40% at 60.3°C and began to lose its activity.

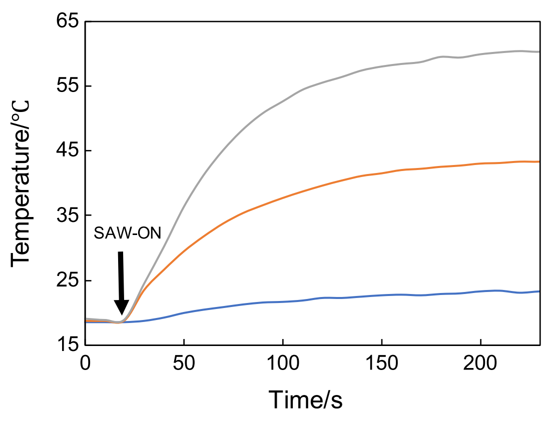

FIGURE 2 Temperature change in the liquid pool with changing power applied for SAW generation. Blue: 0.1 W, Brown: 0.5 W, Gray: 1.0 W

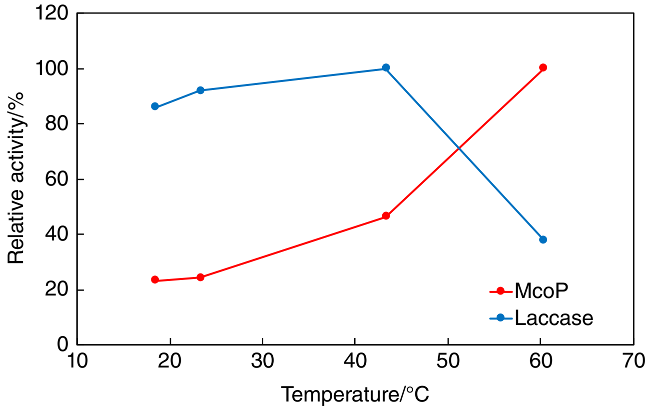

FIGURE 3 Comparison of relative activity of McoP and laccase at each temperature

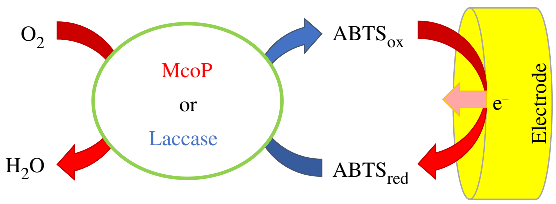

FIGURE 4 Schematic diagram of the electrochemical reaction of McoP or laccase

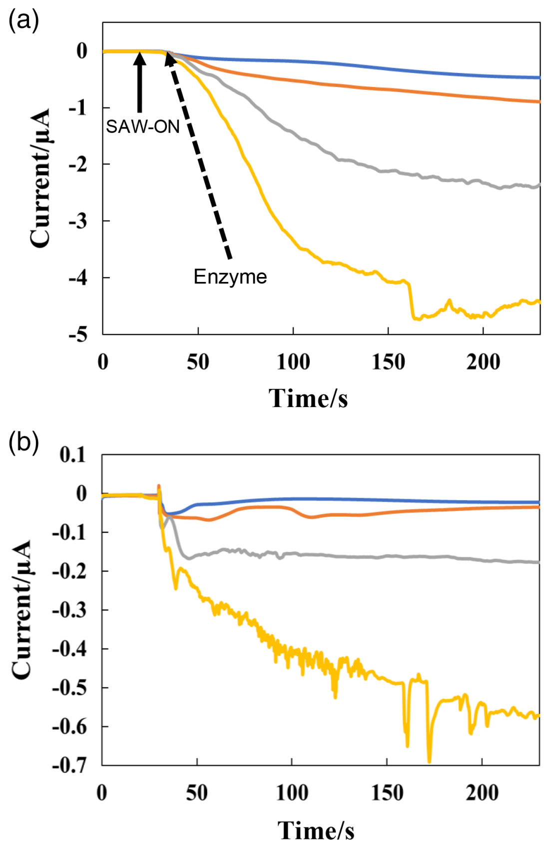

FIGURE 5 Electrochemical reduction current produced by (a) laccase and (b) McoP under different electric power to IDTs: Blue: 0 W, Brown: 0.1 W, Gray: 0.5 W, Yellow: 1.0 W

3.3 | Enzyme electrochemical reaction measurement by SAW agitation

When MCO was characterized electrochemically, ABTS are used as mediators. ABTS was oxidized by the catalytic reaction between O_2 and McoP. Thus, the reduction current was amplified because the oxidized form of ABTS was increased (Figure 4). Figure 5 shows the results of current produced by McoP and laccase under different agitation conditions of the SAWs. In this study, ABTS was dissolved as a mediator, and the reduction currents of McoP and laccase were compared by providing 100 mV constant potential. From 20 s after the potential application, the SAWs were driven to start stirring. After 10 s from the start of stirring, McoP or laccase was dropped into the pool. Figure 5(a) shows the result of electrochemical measurement when laccase was used, and Figure 5(b) shows the result of electrochemical measurement when McoP was used. When McoP was used, the finally obtained current value increased depending on the power supplied to the IDT, the same when using laccase. These results show the facilitated transfer of the enzyme and the substrate to the electrode surface by the stirring effect of the SAW and the promotion of the electrochemical reaction. When the power supplied to the IDT was 1.0 W, and SAW-driven stirring was performed, obtained current was increased 9.4 times using laccase, whereas the final obtained current value was increased by 26 times when using McoP (Table 1). This is because, as shown in Figure 3, the heat-resistant enzyme McoP retained its activity even at high 60°C, while laccase activity reduced by half or even more. Although laccase began to lose activity when the supplied power was 1.0 W, it was still possible to measure the largest current value compared to other supplied powers. This indicates that the effect of increasing the current value by the stirring effect of the SAWs was greater than the decrease in the current value after the inactivation of the enzyme.

TABLE 1 Relationship between SAW-driven temperature change and electrochemical reaction

		Reduction	Current (fold)
Supply power (W)	Temperature (°C)	McoP	Laccase
0.1	23	1.6	1.9
0.5	43	8.2	5.1
1.0	60	26	9.4

4 | CONCLUSIONS

In this study, we evaluated the characteristics of an enzymatic-electrochemical reaction using the stirring effect and heating effect of SAWs and a thermostable enzyme and showed that the McoP, a hyperthermostable enzyme, was suitable for use in a μTAS with

SAW-driven sample stirring. Changes in enzyme activity with changes in the power supplied to the IDT established a correlation between the current production and the enzyme activity. Using a SAW device to electrochemically measure the reaction of a thermostable enzyme, the increase in diffusion coefficient due to increase in the temperature the solution was maximized; therefore, the current value could be increased by taking advantage of the increased diffusion limit. Thus, a high-sensitivity biosensor can be constructed by combining a SAW device and a thermostable enzyme.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

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