Improved Sensitivity of Acetaldehyde Biosensor by Detecting ADH Reverse Reaction-Mediated NADH Fluoro-Quenching for Wine Evaluation

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ABSTRACT: Acetaldehyde (AcH) is found in ambient air, foods, and the living body. This toxic substance is also contained in wine and known as an important ingredient affecting the quality of wine. Herein, we constructed and evaluated two different fiber-optic biosensors for measurement of AcH in the liquid phase (AcH biosensor) using aldehyde dehydrogenase (ALDH) or alcohol dehydrogenase (ADH). The AcH biosensor measured a concentration of AcH using fluorescence intensity of a reduced form of nicotinamide adenine dinucleotide (NADH) that was produced or consumed via catalytic reaction of the respective enzyme. In the AcH measurement system, an ultraviolet light emitting diode (UV-LED) and photomultiplier tube (PMT) were connected to a bifurcated optical fiber and were used to excite and detect NADH. A sensing region was developed using an optical fiber probe and an enzyme-immobilized membrane, buffer pH, and concentrations of a coenzyme in buffer solution for ALDH forward reaction and ADH reverse reaction were optimized, and the dynamic ranges were compared. ADH-mediated AcH biosensor showed higher sensitivity, wider dynamic range (1−500 μM), and capability of rapid measurement (less than 3 min) than ALDH-mediated AcH biosensor (5−200 μM). ADH biosensor also presented a high selectivity and allowed measurement of AcH in 9 different wine samples (5 red and 4 white wines). The determined concentrations were comparable to those measured by NADH absorbance method, which validated the accuracy of the ADH biosensor in AcH measurement.

KEYWORDS: biosensor, acetaldehyde, NADH, alcohol dehydrogenase, aldehyde dehydrogenase, fiber-optic, wine

Acetaldehyde (AcH, CH₃CHO) is one of the volatile chemical compounds (VOCs) that is generally present in the environment. This toxic substance is also produced during the process of ethanol metabolism in the human body. An animal experiment on long-term administration of AcH to rats conducted in 2002 reported a significant increase in malignant tumors and carcinogenicity of AcH. Also, AcH has been connected to DNA toxicity, which can cause various diseases. The International Agency for Research on Cancer (IARC) reported that AcH is highly likely to be carcinogenic to...
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ACSHirth. 12.1.5 from yeast, 20 unit/mg solid, A7011 were purchased from Roche diagnostics (Germany) and Sigma-Aldrich (USA), respectively. Hydrophilic polytetrafluoroethylene (H-PFTE, porosity of 80%, pore size of 0.2 μm, thickness of 80 μm, omnipore membrane filters, HOOWPI425) for a substrate of enzyme immobilization was from Millipore (USA). A polymer immobilizing ADH or ALDH in the H-PFTE membrane, poly[2-methacryloyl oxyethyl phosphorylcholine (MPC)-co-2-ethylhexyl methacrylate (EHMA)] (PMEH), was synthesized in-house by a free radical polymerization method. Reduced (NADH, No. 44327000) and oxidized form (NAD+ No. 44057000) β-nicotinamide adenine dinucleotide were from Oriental Yeast (Japan). Buffer chemicals including acetic acid (99.7%, No. 017-00256), sodium acetate (98.5%, 192-01075), potassium dihydrogen phosphate (99.5%, 169-04245), sodium hydrogen carbonate (99.5%, 191-01305), and sodium carbonate (99.8%, 199-01585), and standard AcH solution (90%, 015-09576) were purchased from Wako (Japan). All of the buffer solutions were prepared with ultrapure water obtained by Mill-Q purification system from Millipore (USA). Chemical substances used for investigating the selectivity of the biosensor were malic acid (99.05%, 135-00562, Wako, Japan), tartaric acid (99.5%, 03045, Yoneyama yakuhin kogyo, Japan), succinic acid (99.5%, 190-04332, Wako, Japan), citric acid (99.5%, 251275, Sigma-Aldrich, USA), glycerol (87%, Kenei, Japan), glucose (98.0%, 049-31165, Wako, Japan), and ethanol (99.5%, 14033-00, Kanto Chemical, Japan). All of the wines were purchased at a local store in Japan.

Construction of Acetaldehyde Measurement System. Figure 1 shows detection principles of AcH for the (a) ALDH-mediated and (b) ADH-mediated AcH biosensors. In the reaction of ALDH, NAD+ is reduced to NADH as an electron acceptor when AcH is oxidized to acetic acid. On the other hand, NADH is oxidized to NAD+ when AcH is reduced to ethanol in the reaction of ADH. The most well-known catalytic reaction of ADH is to oxidize ethanol to produce AcH; this reaction was defined as a forward reaction of ADH. Vice versa, a reaction reducing AcH to produce ethanol was defined as a reverse reaction of ADH in this paper. Incidentally, ADH is not capable of catalyzing to reduce acetic acid to AcH as a reverse reaction according to Black’s research. It is known that NADH used in both enzymatic reactions shows autofluorescence at a wavelength of 490 nm by excitation with ultraviolet (UV) light at a wavelength of 340 nm. In contrast, NAD+ does not exhibit such a fluorescence property.
Therefore, detecting NADH selectively by measuring the fluorescence intensity at 490 nm is feasible. Since the fluorescence intensity of NADH depends on its concentration that is correlated with AcH concentration, it is possible to quantify AcH by measuring the fluorescence intensity of NADH in both enzymatic reactions. ACh biosensor was constructed with a UV light emitting diode (UV-LED, 3 of 335 nm, Sensor Electronic, USA) with a stabilized DC power source (Yokogawa, Japan) as a NADH excitation light source, a photomultiplier tube (PMT, Hamamatsu photonics, Japan) as a fluorescence detector, and a bifurcated optical fiber (Ocean Optics, USA) to connect UV-LED and PMT. Two band-pass filters were used on the UV-LED (BPF_{ex} 340 ± 10 nm, MX0340, Asahi spectra, Japan) and PMT side (BPF_{em} 490 ± 10 nm, MX0490, Asahi spectra, Japan) for getting rid of unnecessary light, which improved the signal-to-noise ratio for photodetection of NADH. An optical fiber probe (F1000–900, Ocean optics, USA) with the enzyme-immobilized membrane was connected to the common end of the bifurcated fiber (Figure 2).

![Figure 2. Schematic illustration of ACh biosensor including a UV-LED, PMT, and optical fiber probe with an enzyme immobilized membrane. Enzyme immobilization for both ALDH and ADH was performed by physical entrapment with PMEH.](image)

The concentration of AcH solution was measured using the ACh biosensor. The NADH absorbance method was used as a comparison, in order to perform calibration curves of ALDH and ADH biosensor for various concentrations (10 nM to 1 mM) of standard ACh solution.

![Table 1. Information of Measured Wine Samples](image)

Table 1. Information of Measured Wine Samples

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<th>ID</th>
<th>type</th>
<th>ethanol conc. (%)</th>
<th>ethanol conc. (M)</th>
<th>additives</th>
<th>pH</th>
<th>pH original</th>
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<tr>
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The self-assembly method was used as a comparison, in order to confirm reliability of the measured concentration by the ACh biosensor. Characteristics of each sample are shown in Table 1. In the preparation of the sample, it has been reported that it is necessary to decolorize a red wine sample if it has an optical density of more than 0.5 at 340 nm. However, when the sample prepared this time was diluted 10-fold, no pretreatment was needed because OD was much less than 0.5 in all samples (Figure S-2). In this experiment, the ACh sensor tip was immersed in 270 μL of NADH coenzyme solution (500 μM in 0.1 M PB, pH 6.5) and then 30 μL of wine was injected after the background was stabilized. Change in fluorescence intensity from the baseline after the sample injection was defined as ΔI, and the concentration of ACh in the wine sample was determined from the calibration curve.
In the NADH absorbance method, first 135 μL of a NADH coenzyme solution (1 mM in 0.1 M PB, pH 6.5) was dispensed into a micro tube, and then 30 μL of wine was added to the tube. Three minutes after vortexing this mixed solution (NADH-wine solution), absorbance at a wavelength of 340 nm (A1) was measured by a spectrophotometer (nanodrop 2000, Thermo Fisher Scientific, USA). Afterward, 135 μL of ADH solution (4.2 units in 0.1 M PB, pH 6.5) was added to the NADH–wine solution and the mixture (NADH–wine–ADH solution) was vortexed and the solution allowed to stand for 10 min. Then, the absorbance of NADH–wine–ADH solution (A2) was measured. Finally, AcH concentration of the wine was determined by substituting the value of A1 – A2 into a calibration curve prepared using a standard AcH solution. In addition, in order to confirm whether it was properly quantified, NADH–wine solution spiked with 100 μM and 500 μM of AcH were prepared, and the absorbance was measured.

RESULTS AND DISCUSSION

Sensor Responses Using ALDH Forward Reaction and ADH Reverse Reaction. At first, it was investigated whether AcH biosensor using ADH or ALDH shows a response to AcH in the liquid phase. Figure 3 shows the responses to AcH, and it was observed that the sensor output before injecting AcH was stable in both biosensors. In the ALDH biosensor, increase in the fluorescence intensity was accompanied by dropping of AcH solution. In contrast, in the ADH biosensor, decrease in the fluorescence intensity was observed. These were attributed to NADH production in ALDH-mediated reaction and consumption in the ADH-mediated reaction, respectively. The output signal ΔI in both sensors were defined as the difference between the average intensity from 2.5 to 3 min after the injection and the baseline. ΔI showed dependence on the concentration of AcH in both sensors, which suggested the possibility of quantifying the AcH concentration based on the fluorescence intensity of NADH produced or consumed by the forward reaction of ALDH and the reverse reaction of ADH.

Optimization of Solution Conditions and Selection of a Suitable Enzyme for AcH Biosensor. Figure 4a shows normalized intensities of ALDH biosensor to 100 μM of AcH in coenzyme solution adjusted to pH range of 3–11. The intensity peaked when using pH 8.0 and drastically decreased at the other pH; thus, it was decided to carry out subsequent experiments at pH 8.0 for ALDH sensor. Similarly, Figure 4b shows the results for the ADH sensor. In the graph, the filled markers show the outputs in the reverse reaction of ADH, and indicated that the reduction of AcH by ADH reached a maximum at pH 6.5. Also, when 100 μM of ethanol was injected to the optimum solution (500 μM NADH in 0.1 M PB, pH 6.5), no output was observed, which suggested that forward reaction of ADH did not occur under this optimum condition of the solution. Although this result was obtained without NAD+ in the buffer solution, even in the measurement of AcH via ADH reverse reaction that produces ethanol and NADH, ADH forward reaction would neither occur nor interfere with the sensor outputs for the following reasons: First, according to Dickenson et al.,39 a product inhibition probability of ADH reverse reaction is 1/7000 of that of ADH forward reaction, which indicates the reverse reaction is unlikely to be affected by ethanol; second, Figure 3b shows that fluorescence intensity was stable after it reached a plateau even when applying a high concentration of AcH (1 mM). Suppose that the forward reaction remarkably occurred, the fluorescence intensity would increase because of production of NADH.

The outputs of the ADH biosensor were kept close to the maximum at a slightly acidic condition (pH 6.0–7.0), while the ALDH decreased by about 20% of the signal at pH 6.0. This result implies that the ADH biosensor is suitable for measurement of AcH in wine because wine is usually slightly acidic (pH of 2.9–3.9).

Here we investigated the influence of coenzyme concentration. In ALDH biosensor, when the concentration of NAD+ was 100 μM, AcH solution could be measured from 1 μM and the output reached saturation at the concentration above 20 μM due to lack of NAD+. In the cases using 500, 1000 μM of NAD+, there was no difference in the dynamic range (5–200 μM); thus we decided to select 500 μM of NAD+ in the ALDH biosensor to reduce the consumption of the coenzyme (Figure S-1a). In ADH biosensor, dynamic range can be adjusted by the concentration of NADH in coenzyme solution (Figure S-1b): dynamic range of 0.5–20 μM (100 μM NADH), 1–500 μM (500 μM NADH), and 20–1000 μM (1000 μM NADH). The lowest concentration of AcH (0.5 μM) was detected by using 100 μM of NADH. The broadest dynamic range was achieved by using 500 μM of NADH; thus we selected 500 μM of NADH in the following experiment since the reported concentrations of AcH in 10-fold diluted wines were 9–481 μM.35

Figure 5 shows calibration curves of ALDH and ADH biosensors for AcH solution obtained under the optimum conditions. A dynamic range of each sensor was determined as the range where the correlation coefficient of a fitting curve is

Figure 4. Dependence of (a) ALDH and (b) ADH activities to AcH on the buffer pH. (▲) acetate buffer, AB; (●) phosphate buffer, PB; (●) carbonate–bicarbonate buffer, CB; (◇)Tris-HCl buffer, TB. Solid and dashed lines in (b) indicate the reverse and forward reactions of ADH, respectively. The forward reaction was examined by injection of ethanol.
over 0.999. ADH biosensor showed a wider dynamic range of 1–500 μM with respect to that of ALDH biosensor (5–200 μM).

**AcH Concentration in Wine.** Figure 6a describes the relative sensor outputs to the 10-fold diluted substances, which exist in approximately 90% of red wine. The output of 160 μM ACh was defined as 100%, and the others were divided by the ACh value. The ADH-mediated ACh biosensor showed an obvious output from the ACh solution, while it was rarely observed from the other substances. It validated a high selectivity of the ADH biosensor to ACh that was based on the substrate specificity of the enzyme. Although, in general, ADH shows activity against various chemical substances that with aldehyde groups, it was reported that about 90% of the substances contained in wine was ACh. Therefore, other aldehydes were not considered in this investigation.

Figure 6b shows the results of quantitative determination of the ACh concentrations in nine different wine samples (5 red and 4 white wines) by the ADH-mediated ACh biosensor. Note that the concentrations in Figure 6b represent those of undiluted wine samples which were obtained by multiplying the experimental data by ten. For comparison, ACh concentrations in the wine samples were also measured by a standard NADH absorbance method (Figure S-3a). As displayed in Figure 6b, ACh concentrations determined by both methods were consistent, which suggested that ethanol in the wine samples did not influence ACh biosensor and the measurement was accurate. ACh concentrations in the red wines were slightly lower than those in the white, which were in good agreement with the previous reports. Reasons for the difference in ACh concentration between red and white wines are mainly related to malolactic fermentation during the production process, which can degrade ACh in wine. Malolactic fermentation is rarely applied to white wine for which a sour taste is preferred since the fermentation converts malic acid with strong sourness to lactic acid, and adds a complexity in taste. On the other hand, malolactic fermentation is routinely used to impart rich flavor to almost all red wines and to improve storage stability. Therefore, red wines tend to show lower ACh concentration than that in white wines.

To further confirm the accuracy of the measurement, ACh concentrations of spiked samples prepared by adding a standard ACh solution of 100 μM and 500 μM to the wine were also measured. The recovery rates were 92% and 94%, and it proved the accuracy of the quantified ACh concentrations by the ADH biosensor. Additionally, high correlation coefficient ($R = 0.999$) and probability ($p < 0.001$) between the results of ADH biosensor and NADH absorbance method were observed (Figure S-4).

These results demonstrated some notable advantages of ACh biosensor with respect to the conventional NADH absorbance method: ACh biosensor was 100-fold more sensitive than the NADH absorbance method as the limit of quantification of conventional NADH absorbance method for ACh was 100 μM (Figure S-3b); the sensing part of ACh biosensor can be miniaturized and is reusable for several measurements with a single enzyme immobilized membrane, which allows saving enzyme consumption; ACh biosensor holds potential for continuous measurement of ACh, which is useful, e.g., for monitoring ACh in wine through the production.

**CONCLUSIONS**

In this study, a fiber-optic biosensor was developed for measurement of ACh in solution by combination of the NADH fluorescence detection system and the enzyme-immobilized membrane that exploited ALDH forward reaction or ADH reverse reaction. It showed a wider dynamic range (1–500 μM) of the ADH-mediated biosensor than that of the ALDH (5–200 μM). Thus, the reverse reaction of ADH was selected to the ACh biosensor. High selectivity of the ADH-mediated ACh biosensor to ACh was validated by the main substances contained in the wine. Measurement of ACh in 9 different wines (5 red and 4 white wines) was also carried out, and accurate quantification by the ADH biosensor was demonstrated. The developed ADH biosensor provides a simpler and more rapid method to quantify ACh concentration in solution than conventional analytical instruments. Furthermore, it is capable of real-time measurement. This novel sensor is not only applicable to analyzing the concentration of ACh in wine but also can be used for determination of ACh in body fluid for medical and health care related applications.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.7b00184.
Additional figures: Influence of coenzyme concentration; Optical densities; Representative absorbance spectra; Correlation of AcH concentration (PDF)

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**Notes**
The authors declare no competing financial interest.

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