An Interference-Free, Amperometric Biosensor for Acetic Acid Based on a Tri-enzyme/polydimethylsiloxane-bilayer Membrane

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An amperometric acetate-sensing electrode with high performance characteristics, such as high selectivity (e.g., free from the interference by ethanol, L-cysteine and hydrogen peroxide) and high stability (usable for a month), was prepared by immobilizing acetate kinase, pyruvate kinase and pyruvate oxidase on a polydimethylsiloxane-coated oxygen electrode.

The accurate and rapid determination of acetic acid is necessary in industrial food laboratories: acetic acid is a component of fermentation products such as wine and soy sauce. Biosensors can accomplish this function. A few papers have so far been presented on acetate biosensors. Nanjo and Guilbault[1] described the construction of an alcohol oxidase-based electrode, and Hikuma et al.[2] reported a microbial electrode using *Trichosporon brassicae*. However, each sensor exhibited a poor selectivity for acetic acid: the addition of ethanol gave a much larger response than the same concentration of the analyte,[1,2] so that the determination of acetic acid in such ethanol-containing food samples was impracticable.

We have recently developed an amperometric ADP sensor by using the enzyme pair of pyruvate kinase (PK, EC 2.7.1.40) and pyruvate oxidase (PyOx, EC 1.2.3.3),[3] whose catalyzing reactions are:

$$\text{ADP + phosphoenolpyruvate} \xrightarrow{\text{PK}} \text{ATP + pyruvate}$$

$$\text{pyruvate + } HPO_4^{2-} + H_2O + O_2 \xrightarrow{\text{PyOx}} \text{acetyl phosphate + } HCO_3^- + H_2O_2.$$

In this work, we prepare a new acetate biosensor by coupling the ADP-sensing system with phospho-transferring reaction catalyzed by acetate kinase (AK, EC 2.7.2.1):

$$\text{ATP + acetate} \xrightarrow{\text{AK}} \text{ADP + acetyl phosphate.}$$

Although ADP, pyruvic acid and propionic acid (another substrate of AK)[4] are expected to give positive errors in measuring acetic acid by the AK/PK/PyOx-system, the concentrations of these compounds in food samples are quite low. As the base transducer, we have employed an electrode coated with polydimethylsiloxane (PDMS) for detecting oxygen. The cathodic detection can essentially be free from interference from oxidizable species (e.g., L-ascorbic acid and L-cysteine) in the sample to be measured. Further, the permselectivity of PDMS made it possible to monitor the enzymatic oxygen consumption rate without interference from the hydrogen peroxide produced.[5] Such a polymer-coated electrode can be prepared easily and cheaply, as compared with conventional Clark-type oxygen electrodes. Thus the tri-enzyme/PDMS-bilayer-based electrode would be highly suitable for the accurate determination of acetic acid in food samples.

A platinum disc electrode (d., 1.6 mm) was dipped into an emulsion of PDMS (BY22-826, Toray Dow Corning Silicone), which was diluted to 5%(w/v) with water just before the coating process, and allowed to dry. The thickness of the PDMS layer was ca. 20 μm. A tri-enzyme membrane was prepared from a mixture of a photo-crosslinkable poly(vinyl alcohol) (PVA-SbQ,[6] 11%(w/v) aqueous solution) and a 0.02 M phosphate buffer (pH 7) containing AK (from *Bacillus stearothermophilus*, 770 U mg^{-1}, Sigma), PK (from rabbit muscle, 380 U mg^{-1}, Sigma) and PyOx (from *Aerococcus viridans*, 47 U mg^{-1}, Asahi Chemical Industry), 1:3 by weight, according to the procedure described elsewhere.[3] Here, the concentration of each enzyme in the PVA-SbQ/buffer mixture was 0.5%. The enzyme membrane prepared (thickness, 30 μm) was placed on the PDMS layer and covered with a polyester mesh (100 mesh). The mesh was held in place with a rubber ring so that the tri-enzyme membrane was directly in contact with the PDMS-coated electrode surface.

The test solution used was an air-saturated 0.1 M phosphate buffer (pH 7.0, 20 ml) containing 1 mM ATP, 1 mM phosphoenolpyruvate, 5 mM $MgCl_2$, 0.1 mM tyamine pyrophosphate and 0.02 mM FAD. Here, Mg^{2+} was the activator of AK, PK and PyOx; tyamine pyrophosphate and FAD were activators of PyOx. The solution was stirred with a magnetic bar, and its temperature was kept at 25.0 ± 0.2 °C. The potential of the enzyme electrode was set at –0.4 V vs Ag/AgCl (saturated with KCl).

Figure 1 shows a current–time curve obtained upon the addition of acetic acid (a,c), propionic acid (b), ethanol (d), hydrogen peroxide (e), L-cysteine (f) and L-ascorbic acid (g). The cathodic current decreased after the addition of acetic acid or propionic acid and reached in the steady state within 40 s. The steady-state current decrease for acetic acid was proportional to the analyte concentration up to 0.5 mM. The detection limit was 5 μM (signal-to-noise ratio, 5). The relative standard deviation for ten successive measurements of 0.1 mM acetic acid was 1.8%.

As shown in Figure 1, the addition of L-ascorbic acid brought about a decrease in the cathodic current. A similar L-ascorbate response was observed on an enzyme-free PDMS-coated electrode. These results suggest that the current decrease is not caused by the enzymatic oxidation of L-ascorbic acid but by the chemical reaction between the species and oxygen. However, the magnitude of the L-ascorbate response was small: the ratio of the response for L-ascorbic acid to that for the same concentration of acetic acid was less than 0.01. It has been shown that electrode current did not change at all upon the addition of ethanol, hydrogen peroxide and L-cysteine. This suggests that the tri-enzyme electrode can be used for determining acetic acid in ethanol-containing food samples.

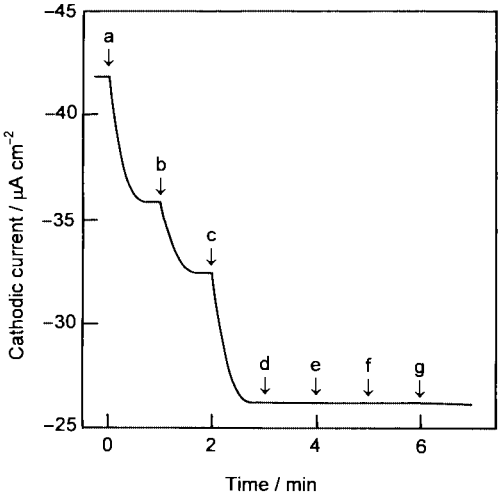

Figure 1. Current–time curve for the tri-enzyme electrode obtained on the addition of 0. 1 mM acetic acid (a,c), 0.1 mM propionic acid (b), 0.1 mM ethanol (d), 0.1 mM hydrogen peroxide (e), 0.1 mM L-cysteine (f) and 0.1 mM L-ascorbic acid (g).

Table 1. Comparison of results obtained for acetic acid in food samples by different methods

Sample	Acetate concentration/mM	
	Proposed method	F-kit method
Wine 1	6.25	6.01
Wine 2	9.15	9.03
Wine 3	11.2	11.5
Wine 4	11.3	11.0
Soy sauce 1	27.3	24.2
Soy sauce 2	21.3	21.2
Vinegar	658	674

Table 1 shows the results for the determination of acetic acid in wines, soy sauces and vinegar. The samples were used without any pretreatment. The sample volumes were 100, 50 and 2 μL for wines, soy sauces and vinegar, respectively. The results were compared with those given by the F-kit method. The kit (Boehringer Mannheim) uses the enzyme system of acetyl-CoA synthetase (EC 6.2.1.1), arylamine acetyltransferase (EC 2.3.1.5) and malate dehydrogenase (EC 1.1.1.37). The agreement was excellent for these food samples; e.g., for the seven samples given in Table 1, the regression equation between the results obtained by the F-kit method (x) and those by the present sensor method (y) was $y = 0.9301 + 0.9750x$ and the correlation coefficient was 0.999. These results clearly show that the present tri-enzyme electrode is useful for the simple and accurate determination of acetic acid in food samples.

The long-term stability of the tri-enzyme electrode was examined; the measurements of current response for 0.1 mM acetic acid were carried out ten times a day for one month. The average value of the electrode response did not change for two weeks (for ca. 100 assays). The electrode response gradually decreased after two weeks, but it was ca. 90% of the initial value even on the 30th day (after 200 assays). The present tri-enzyme electrode could be used for the determination of acetic acid for a long time.

PyOx from *Pediococcus* sp. has widely been used for preparing pyruvate- and phosphate-sensing electrodes.[7] However, the poor stability of the enzyme often resulted in a rapid decrease in the electrode response. We[3] have previously found out that the use of PyOx from *Aerococcus viridans*, instead of the same enzyme from *Pediococcus* sp., was greatly effective for improving the stability of the enzyme-based sensors. Further, our preliminary experiments have indicated that AK from a thermophile, *Bacillus stearothermophilus*, was useful in terms of the stability as compared with AK from *Escherichia coli* (data not shown). Thus the high stability of the present acetate sensor was achieved by the favorable choice of the enzymes.

In conclusion, we have first developed an acetate biosensor with high performance characteristics, such as high selectivity (e.g., free from the interference by ethanol, L-ascorbic acid and hydrogen peroxide) and high stability (usable at least for a month).

References and Notes

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