Flow-Injection Analysis of Ethanol Based on Oxygen Consumption Using Alcohol Dehydrogenase and NADH Oxidase from Bacillus licheniformis

Hiroyuki Ukeda*, Yoshihiro Fujita*, Masayoshi Sawamura*, Hirozo Kusunose* and Yasuhiro Tazuke**

*Department of Bioresources Science, Faculty of Agriculture, Kochi University, Monobe B-200, Nankoku 783, Japan
**Kyoto Research Laboratories, Marukin Shoyu Co. Ltd., Todo, Uji, Kyoto 611, Japan

A flow-injection analysis system for the determination of ethanol was developed incorporating an oxygen electrode and an enzyme reactor packed with Sepharose 4B on which alcohol dehydrogenase (ADH) and NADH oxidase (NOD) from Bacillus licheniformis were co-immobilized. Although the response of the system depended on the concentration of FAD added into a carrier solution, it was linearly related to the ethanol concentration over the range 0.25 – 1.0 mM, even in the absence of FAD, by controlling the immobilized amount of ADH and NOD. The linear relationship between the ethanol concentration and the response in the presence of 60 sM FAD was observed over the range 0.1 – 1.5 mM. When the system was applied to the determination of ethanol in various kinds of alcoholic beverages, the results obtained under both conditions were comparable to those obtained by the F-kit method.

Keywords Oxygen electrode, NADH oxidase, flow injection analysis, ethanol determination

NAD-requiring dehydrogenases are potentially useful catalysts in analytical chemistry. An attractive use of this class of enzyme is to regenerate NADH, formed in the reaction, to NAD with dissolved oxygen. This regeneration system of NADH makes it possible to handle the enzyme as “oxidase-like” and to use an oxygen electrode in the determination of substrates. The use of a Clark oxygen electrode as a detector is especially advantageous for the analysis of foods which contain a relatively high content of electroactive species, because the work electrode is protected against passivation in the solution by a gas-permeable membrane.

For detecting the oxidation reaction of NADH with the oxygen electrode, the following two methods have been proposed: the combined use of enzyme diaphorase and the mediator vitamin K₃ and the use of the enzyme NADH oxidase. The first method reported by the author has been applied to a determination of ethanol and L-malate and the feasibility was shown by its applicability to practical samples. However, the use of vitamin K₃ in oxidized form was unfavorable to SH-enzyme sensitive to oxidation. Mizutani et al. have applied NADH oxidase from Bacillus megaterium for the detection of NADH with an oxygen electrode. The activity of the enzyme remarkably depended on an exogenous addition of FAD as an activator, and a high concentration (0.2 mM) was required in the reaction. The addition of FAD in such a high concentration should be avoided based on a consideration of economical views and the possibility of a side reaction between FAD and some redox compounds in the foods.

The present paper describes a determination system of ethanol with a Clark-type oxygen electrode based on the combined use of NADH oxidase from Bacillus licheniformis and alcohol dehydrogenases (Fig. 1). The aim of this investigation was to construct a determination system of a substrate for dehydrogenase without requiring an exogenous addition of FAD.

**Experimental**

**Reagents**
CNBr-activated Sepharose 4B was obtained from Pharmacia LKB. NAD was purchased from Kohjin Co. Alcohol dehydrogenase (ADH, EC 1.1.1.1; from yeast, 142 IU/mg powder) was obtained from Oriental...
Yeast Co. and NADH oxidase (NOD, EC number not available; 60 units/mg protein) from Bacillus licheniformis was prepared by Marukin Shoyu Co; it is also presently available from Nacalai Tesque Co. Flavine adenine dinucleotide (FAD) was purchased from Nacalai Tesque Co. All other chemicals were of analytical grade and were used without further purification. Distilled water was used for all procedures.

Enzyme immobilization

ADH (1000 IU) and NOD (15 or 30 units) were immobilized on CNBr-activated Sepharose 4B (0.20 g dry weight), as reported previously, and was packed into an acrylic tube (3 mm i.d.×6.4 cm). The enzyme reactor packed with the gel in which 1000 IU of ADH and 15 or 30 units of NOD were immobilized were called Reactor 1 and Reactor 2, respectively. The enzyme reactor was stored in 0.1 M phosphate buffer (pH 8.0) at 5°C.

Flow system

A schematic diagram of the flow system is shown in Fig. 2. A Teflon tube (0.86 mm i.d. and 1.4 mm o.d.) was used throughout the flow system. The standard solution of ethanol and sample solutions were diluted with a 0.1 M phosphate buffer (pH 8.0). These solutions were mixed with the equivalent volume of a 5 mM NAD solution prepared with the same buffer and the mixed solution (20 µl) was injected into a carrier stream with a sample injector (Rheodyne, Model 9125). The carrier was pumped with a peristaltic pump (Gilson, Minipuls 2). The injected sample was pumped through the enzyme reactor to a flow-through cell (Denki Kagaku Keiki Co., FLC-41) equipped with a Clark-type oxygen electrode (Denki Kagaku Keiki Co.). The potential applied at the oxygen electrode was −0.7 V, amperometric measurements being made with a potentiostat (Denki Kagaku Keiki Co., JOL-50). The peak height was recorded as the response of a recorder (Graphitec, SR 6211). In the present study, the concentration of ethanol in the standard and sample solutions was indicated as that in the solution before being mixed with the NAD solution. A phosphate buffer (0.1 M, pH 8.0) containing or not containing FAD served as a carrier solution, unless otherwise mentioned. The determinations were all carried out at 26±1°C.

Results

In the reaction of NOD, generally, FAD has been added to the assay mixture as an activator of the enzyme. The activity of NOD from Bacillus licheniformis used in this work is reported to increase to 20-fold due to the addition of FAD at a concentration of 30 µM. This may mean that the affinity of the enzyme with FAD is lower compared with that of other flavoproteins, such as glucose oxidase and diaphorase. Therefore, we first examined the effect of the FAD concentration in the carrier solution on the response for ethanol using the two kinds of enzyme reactors (Fig. 3). As shown in the figure, a rapid increase in the response was observed up to 30 µM and a tendency to level off was recognized above this concentration in both enzyme columns. In the presence of FAD (30 µM), Reactor 2 gave a slightly bigger response compared with Reactor 1. In the absence of FAD, however, the response of Reactor 2 was about 1.5-times that of Reactor 1. This result means that the dependence of FAD on the response can be controlled by adjusting the amount of immobilized ADH and NOD and that the use of a higher amount of NOD against ADH may permit one to construct an analytical system without requiring an exogenous addition of FAD. In order to examine this possibility, we have used not only a carrier solution containing a high concentration of FAD (60 µM), but also that not containing FAD in a subsequent experiment.

Figure 4 depicts the effect of the concentration of NAD on the response. At higher concentrations of NAD than 3 mM, there was no dependency of the concentration on the response. Therefore, we chose 5 mM as the

---

**Fig. 2** Flow system for the determination of ethanol. POT, potentiostat; REC, recorder.

**Fig. 3** Effect of the FAD concentration added into the carrier solution on the response for 1 mM ethanol. Reactor 1, ■; Reactor 2, ●.
optimum concentration in further experiments.

When the flow rate was changed in the 0.25 to 1.0 ml/min range, both the response and the time for base-line reversion decreased along with an increase in the flow rate (Fig. 5). The choice of the flow rate involves a compromise between the sensitivity and the sample output rate. A flow rate of 0.5 ml/min was used in this experiment, considering the relatively high response and the short sample output time.

The dependence of the pH of the carrier buffer on the response was examined in the pH range 7 to 8.5. The maximum response for 1 mM ethanol was observed at around pH 8 in spite of the presence or absence of FAD in the carrier solution. The pH was located at the middle between the optimum of NOD (pH 6.5 – 7.5) and ADH (pH 9.0).

Under the conditions described above and in both of the reactors, a linear relationship between the ethanol concentration and the response was recognized in the 0.1 – 1.5 mM and 0.25 – 1.0 mM ranges in the presence and absence of FAD, respectively (Fig. 6). The relative standard deviation for ten successive determinations at 2.5 mM level was better than 0.5%, and the sampling frequency was about 25 samples/h (Fig. 6).

Table 1 shows the results for a determination of ethanol in some kinds of alcoholic beverages. These results are compared to those obtained using the F-kit method. It should be noted that, upon no addition of FAD, the results similarly showed a good agreement with those using the F-kit method. This means, therefore, that Reactor 2 is applicable to the determination of ethanol, even in the absence of FAD.

The stability of the immobilized enzymes was evaluated for 30 d. The response for 2.5 mM ethanol did not decrease, suggesting that the immobilized enzymes had good stability. In our previous paper diaphorase and vitamin K₃ were used in the oxidation reaction of NADH with oxygen, and the determination
system of ethanol was constructed by a combination of immobilized ADH. The activity decreased to 40% of the first one after 8 d. Therefore, the conditions used in this paper are suggested to be much milder for ADH, being sensitive to an oxidizing reagent.

**Discussion**

NOD has been purified from *Brevibacterium ammoniagenes*, *Thermus thermophilus*, *Thermus aquaticus*, *Bacillus licheniformis*, and *Bacillus megaterium*. Murachi et al. developed a highly sensitive FIA system for chemiluminometric determination of NADH using NOD from *B. ammoniagenes*. In the method, FAD was not added to the carrier solution. Mizutani et al. developed enzyme electrodes based on the use of an oxygen electrode for the determination of NADH and malate using NOD from *B. megaterium*, in which a high concentration of FAD (0.2 mM) had to be added to the assay solution. Dremel et al. fluorometrically compared the activity of NOD from *B. licheniformis* with that from *T. thermophilus* and *T. aquaticus* in the presence or absence of FAD and mentioned that only NOD from *B. licheniformis* showed an enough activity for the detection of NADH in the absence of FAD. In the present method in which an oxygen electrode was utilized in the detection of ethanol, the use of NOD from *B. licheniformis* gave a good quantitative response for ethanol and permitted a determination of the ethanol content in various kinds of alcoholic beverages, even in the absence of FAD. Although no experiments for making comparisons with other NOD were carried out, the results obtained in this paper would result from the property of NOD from *B. licheniformis*, judging from the result reported by Dremel et al.

Several types of enzyme sensor systems for the determination of ethanol have been developed and applied to control the fermentation process and the quality of alcoholic beverages. Three ethanol-converting enzymes have been used: alcohol dehydrogenase (alcohol: NAD oxidoreductase, EC 1.1.1.1), alcohol dehydrogenase (alcohol: acceptor oxidoreductase, EC 1.1.99), and alcohol oxidase (alcohol: oxygen oxidoreductase, EC 1.1.3.13). Generally, alcohol oxidase shows a low substrate specificity and it could restrict the applicability of the enzyme. Since alcohol dehydrogenase is more specific for ethanol compared with alcohol oxidase, the use of dehydrogenases may be more advantageous in the analysis of ethanol in fermentation broth and foods comprising many components. However, serious interferences by electroactive species, such as ascorbic acid, are often observed in the amperometric detection of the reaction. In order to overcome the problem, a more complicated detection system should be utilized that can correct the effect of the interfering substances. The use of an oxygen electrode for detecting the dehydrogenase reaction enables one to minimize interference by the electroactive species, because the Teflon membrane on the electrode prevents the species from contacting the working electrode. Furthermore, NOD from *B. licheniformis* showed a low dependence on the exogeneous addition of FAD. This can also contribute to a lowering of the possibility of interference by the electroactive species.

The FIA systems for various analytes could be developed by coupling NOD with NAD-dependent dehydrogenases: over 250 dehydrogenases use NAD as the cofactor for the substrate determination. The low dependence of NOD from *B. licheniformis* on FAD and the high stability of the enzyme have good promise for the further development of an FIA system comprising dehydrogenases and NOD.

**References**


(Received July 12, 1993)

(accepted October 7, 1993)