Fluorometric Determination of Ethanol in Liquor Samples by Flow-Injection Analysis Using an Immobilized Enzyme-Reactor Column with Packing Prepared by Coupling Alcohol Oxidase and Peroxidase onto Chitosan Beads

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A flow-injection system was developed for the determination of ethanol with an immobilized enzyme-reactor column. This system, which consisted of a flow-injection system packed with alcohol oxidase and horseradish peroxidase immobilized onto chitosan beads, and a fluorometric detector, was applied to the determination of ethanol in liquor samples. Under the recommended conditions, the ethanol, which was present in the pretreated samples, was converted to hydrogen peroxide when it was passed through the immobilized alcohol oxidase (AOD) column with 0.1 mol/dm³ phosphate buffer (pH 7.0). A sample can be analyzed with this system in ≈10 min. The calibration curve for ethanol was linear from 2.0 to 0.1 mg/dm³. The determination limit, which was defined by the difference between the sample peak and blank peak, was estimated to be 50 μg/dm³ for ethanol. Interferences from some substances present in actual liquor samples decreased the analytical response and activity of the immobilized AOD-reactor column, but they were removed by dilution and pretreatment with an octyldecysilane cartridge.

Enzymatic assays are based on the use of commercially available alcohol dehydrogenase (ADH; alcohol:NAD⁺ oxidoreductase; Enzyme Commission [EC] 1.1.1.1) or alcohol oxidase (AOD; alcohol:oxygen oxidoreductase; EC 1.1.3.13). The first enzyme catalyzes the oxidation of ethanol to acetaldehyde, and spectrophotometric measurements are made of the reduced coenzyme, NADH (reduced nicotinamide adenine dinucleotide). The assay has been applied with some analytical techniques, such as differential amperometric detection and flow-injection analysis (FIA) with spectrophotometry or fluorometry (18–21). Although AOD as a second enzyme is commercially available, studies using the enzyme are few, because NADH produced by the ADH catalyzing reaction is easily detected. In recent studies, it has been noted that NADH detection is influenced by some coexisting substances and that hydrogen peroxide, which is produced by the AOD catalyzing reaction, can be detected with greater sensitivity than NADH (16, 22). Therefore, an analytical method based on the reaction catalyzed by AOD has more significance. However, because AOD is a comparatively unstable enzyme, the activity of the enzyme immobilized on a microplate cannot be maintained for about 10 days (22).
In our previous paper (11, 12), we reported an FIA system with an immobilized enzyme-reactor column that was prepared by a glutaraldehyde bridge reaction between the enzyme and chitosan beads as a carrier. In subsequent work the enzyme was immobilized onto chitosan beads under milder conditions, and the resulting immobilized enzyme was kept at higher activity. The goal of the work described in this paper was to immobilize AOD onto chitosan beads as a carrier and apply it to the determination of ethanol in spirits by FIA with the immobilized enzyme column and fluorescence detection.

Experimental

Apparatus

(a) Spectrofluorometer.—Shimadzu (Kyoto, Japan) RF-535 spectrofluorometer equipped with a quartz flow cell (12 µL). Fluorescence intensity was recorded by a Fantos Unicorder C-228 recorder.

(b) Spectrophotometer.—JASCO V-530 UV-VIS.

(c) pH meter.—TOA Electronics (Tokyo, Japan) Model HM-30S.

(d) Circulator.—The activities of the immobilized enzymes were measured at 25°C, and temperature was maintained by using a Model CTE-42W Yamato-Komatsu Coolmatics circulator (Tokyo, Japan).

(e) Shaker.—The immobilized enzyme was prepared with a Model V-DN Iwaki KM-Shaker (Tokyo, Japan) which was used at maximum speed.

(f) Pumps.—Nihon Seimitsu Mini-Chemical Pumps Models NP-EX3U and NP-KK-110U (Tokyo, Japan) were used for FIA.

(g) Column.—The immobilized enzyme column was a plastic minicolumn (50 × 5 mm id) packed with 0.8 g peroxidase or AOD immobilized onto chitosan beads.

(h) Cartridge.—A Waters (Milford, MA) Sep-Pak Plus C18 cartridge was used for sample pretreatment.

Reagents

(a) 3-(p-Hydroxyphenyl)propionic acid (HPPA) di guaiacol.—Wako Pure Chemical Co. Ltd. (Osaka, Japan). Used without any further purification. HPPA was dissolved in 0.3 mol/dm³ tris(hydroxymethyl)aminomethane (Tris; pH 7) to give a 2.3 × 10⁻² mol/dm³ solution.

(b) Glutaraldehyde solution.—0.1, v/v; dissolved it in 0.3 mol/dm³ Tris, the pH of which was adjusted to 8 or 10 with nitric acid.

(c) Phosphate buffer solution (pH 7.0).—Obtained by mixing 0.1 mol/dm³ dihydrogen phosphate and monohydrogen phosphate in a 1:2 ratio.

(d) Horseradish peroxidase (POD; EC 1.11.1.7).—With a specific activity of 257 U/mg, used for preparation of the immobilized enzyme (Wako Pure Chemical Co. Ltd) commercially available for biochemical experiments. The POD solution was freshly prepared from solid enzyme, because the activity of an enzyme in solution cannot be maintained within a short time. The concentration of POD dissolved in 0.1 mol/dm³ phosphate buffer (pH 7) was determined by measuring the absorbance at 400 nm.

(e) AOD (EC 1.1.3.13).—With a specific activity of 1000 U/cm³, used for preparation of the immobilized enzyme (Sigma, St. Louis, MO). The AOD working solution was freshly prepared from the commercial enzyme solution, because the enzyme activity was unstable. The activity of AOD dissolved in 0.1 mol/dm³ phosphate buffer (pH 7) was diluted from the pipetted volumes.

(f) Chitosan beads.—Chitopearl 3503, Φ:300 µm. Used as the immobilizing carrier without any particular treatment (Fujifilm; Tokyo, Japan).

(g) Preserving solution for the immobilized POD.—Obtained by dissolving 1.0 g bovine serum albumin (BSA), 0.88 g sodium chloride, and 1.2 g Tris in 100 mL water. The preserving solution for the immobilized AOD was 0.1 mol/dm³ phosphate buffer solution (pH 7).
Figure 2. Scheme of the immobilized enzyme reaction.
Figure 3. Effect of pH on the activity of the immobilized AOD. Concentration of glutaraldehyde, 0.1%; time of reaction between glutaraldehyde and chitosan, 20 min; time of reaction between chitosan and AOD, 10 min; AOD activity, 100 U; chitosan beads, 2.0 g.

All other chemicals were analytical-reagent grade (Wako Pure Chemical Co. Ltd.).

All solutions, except for a fluorometric indicator solution, were prepared with ultrapure water obtained by using a Milli-Q SP Reagent Water System (Millipore Corp., Bedford, MA). A fluorometric indicator solution was prepared with water, which was distilled as ultrapure water in a borosilicate glass still.

**Preparation and Measurement of the Activity of the Immobilized Enzyme**

The immobilized enzyme was prepared by the glutaraldehyde bridging method. The immobilized POD was prepared as described previously (10). Approximately 2 g chitosan beads were placed in a beaker, and 10 mL 0.3% (v/v) glutaraldehyde in 0.3 mol/dm³ Tris, the pH of which was adjusted to 8 with nitric acid, was added, and the contents of the beaker were stirred for 1 h. Then 5 mL 2 × 10⁻³ mol/dm³ borate solution was added, and the contents of the beaker were again stirred for 1 h. The enzyme solution was added to the reaction mixture, which was again stirred for 1 h. After the chitosan beads were washed with water, all these procedures were repeated. The resulting chitosan beads were used as the POD immobilized enzyme (POD-IE) and were stored in a refrigerator at 4°C in the preserving solution.

To prepare the immobilized AOD ca 2 g chitosan beads were placed in a glass centrifuge tube, 10 mL glutaraldehyde solution was added to the tube, and the tube was shaken for 20 min. Then 10 mL AOD solution containing AOD at a concentration of 100 U/mL in phosphate buffer was added to the tube, which was shaken for 10 min. After the beads were washed with water, the resulting chitosan beads were used as the AOD-immobilized enzyme (AOD-IE) and were stored in a refrigerator at 4°C in the preserving solution.

The activity of the POD-IE was measured as described previously (10). The activity of the AOD-IE was measured by a standard method for POD with some modifications. In the measurement of POD activity the rate of the degradation of hydrogen peroxide catalyzed by POD was measured by using guaiacol as an indicator. By strict definition, the activity of AOD was measured from the variation in ethanol concentration/min that was induced by AOD. However, the activity of AOD was usually measured from the concentration of hydrogen peroxide, which was produced by AOD for an immediate reaction with HPPA that was catalyzed by excess POD. In the measurement of AOD activity, 2,2'-azino-di(3-ethylbenzothiazoline-6-sulfonic) (ABTS) is usually used as an indicator (23). However, it was too high in sensitivity to measure the activity of the prepared AOD-IE. Although the sensitivity of guaiacol was lower than that of ABTS, guaiacol whose molecular absorption coefficient was clear, was adopted as the indicator. The concentrations of other substances used in the measurement of AOD activity were the same as those used in the standard method.

Approximately 0.3 g (wt weight) AOD-IE was placed in a 100 mL flask; 17.0 mL 0.1 mol/dm³ phosphate buffer (pH 7), 1.0 mL 6 × 10⁻³ mol/dm³ guaiacol, and 1.0 mL of about 100 mg/dm³ POD were added to the flask. After the suspension was allowed to stand at 25°C for 45 min, 6.6 g/dm³ methanol was added to the mixture, and the whole solution was stirred continuously. At 1.5 min intervals, a 3 mL aliquot of the reaction mixture was placed in a quartz cell, and the absorbance was measured at 436 nm. The solution was then returned to the reaction mixture to maintain the volume of the reaction mixture. After the measurements were made, the AOD-IE was collected and dried in an oven at 105°C for 6 h. The dry weight of the AOD-IE was measured to estimate the activity. The reaction rate was estimated from the difference in the absorbance fluctuation per minute at 436 nm, and the amount of methanol reacted was then estimated in micromoles per minute. As a blank test, the same procedure was performed with water instead of methanol.

The activity per unit weight of the AOD-IE can be calculated from the U value and the dry weight of the AOD-IE used.
<table>
<thead>
<tr>
<th>Species</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Super pure water</td>
<td>47.8</td>
</tr>
<tr>
<td>0.1 mol/dm³ Phosphate buffer</td>
<td>100</td>
</tr>
<tr>
<td>0.16 mol/dm³ NaCl</td>
<td>55.2</td>
</tr>
<tr>
<td>Preserving solution for POD (1% BSA)</td>
<td>13.0</td>
</tr>
<tr>
<td>-0.15 mol/dm³ NaCl + 0.1 mol/dm³ Tris</td>
<td></td>
</tr>
<tr>
<td>Preserving solution for GOD (1% BSA)</td>
<td>65.3</td>
</tr>
</tbody>
</table>

* Measured after 1 week in preserving solution.

### Analytical Procedure for the Determination of Ethanol by FIA

A flow diagram of the proposed method is shown in Figure 1. An aliquot of the sample solution (1.3 mL) was injected into the 0.1 mol/dm³ phosphate buffer (pH 7.0) carrier stream through an injection valve (Rheodyne 7125, Cotati, CA). In the AOD-IE column, alcohol in the sample solution was converted to hydrogen peroxide and aldehyde. Then, this stream was mixed into an HPPA solution as a fluorometric indicator. The mixture reacted in the POD-IE column to produce a fluorescent species. The fluorescence intensity was then measured at an excitation wavelength of 305 nm and an emission wavelength of 405 nm. The concentrations of the alcohol were determined from a calibration graph, which was prepared with the peak heights obtained by using standard ethanol solutions. For the determination of ethanol in actual samples, pretreated as described in Application to Actual Samples, solutions were injected into the FIA system through an octyldecysilane (ODS) cartridge. The flow rates of the carrier and the indicator stream were identical (0.4 mL/min).

### Results and Discussion

#### Immobilized Enzyme

The immobilizing reaction was based on the glutaraldehyde bridging method shown in Figure 2. In our previous paper, the reaction scheme was the same (10). However, the development of an analytical method for the determination of borate, which was based on the formation of a Schiff base generated by the reaction between an amino group and an aldehyde group, showed the possibility of the immobilized reaction. The catalytic effect of boron on the activity of an immobilized enzyme depended on the enzyme used. Although POD and glucose oxidase (GOD), which were used in our previous work (11, 12), influenced the catalytic effect of boron so that the activities of the POD-IE and GOD-IE were improved by the addition of borate solution to the immobilizing reaction mixture, AOD was not influenced by it. In this study, the suitable conditions of the immobilizing reaction, such as the concentration of glutaraldehyde, the reaction time, and the activity of AOD, were examined.

The activity was reduced for the sake of polymerization between the the enzyme and glutaraldehyde in solution at glutaraldehyde concentrations of >0.1%.

The suitable reaction pH for the immobilization of AOD was 10, as shown in Figure 3. In our previous work (10), the suitable reaction pH was 8, and the activity of the immobilized enzyme was reduced beyond that pH because the structure of the enzyme might be changed. Although the activity of AOD-IE was reduced when the alkaline carrier was used in the flow-injection system with AOD-IE, it was increased when alkaline medium was used in the immobilization reaction. On the other hand, investigation of an analytical method for the determination of borate showed that the suitable pH for the reaction producing a Schiff base was 10. These results suggest that the immobilization of AOD is influenced more by the production of Schiff base than by the structure of AOD.

Because the activity of AOD-IE was reduced when the time of reaction between glutaraldehyde and chitosan was >20 min, a reaction time of 20 min was selected. It was thought that beyond 20 min the aldehyde group in glutaraldehyde might be oxidized to carboxylic acid by a dissolved oxygen so that it could not react with an amino group in chitosan. Consequently, the activity of AOD-IE would be decreased.

The activity of AOD-IE was proportional to the activity of AOD used. Because the stoichiometric conversion of ethanol to hydrogen peroxide for up to 2.0 mol/dm³ ethanol was possi-
AOD–IE was prepared with 100 U AOD/g carrier. Therefore, AOD–IE prepared with >100 U AOD/g carrier was used in the flow-injection system.

To maintain the activity of AOD–IE, various solutions listed in Table 1, were examined as preserving solutions for AOD–IE. In the case of POD–IE or GOD–IE (11, 12), a preserving solution that included BSA was the most suitable, whereas 0.1 mol/dm³ phosphate buffer solution (pH 7) was the most suitable for AOD–IE. On the basis of the results, 0.1 mol/dm³ phosphate buffer solution (pH 7) was used as the preserving solution for AOD–IE, and the column packed with AOD–IE was stored in a refrigerator after use.

**Analytical Conditions for FIA**

The manifold design was chosen from results of previous work (12) involving techniques that could use 2 immobilized enzymes. The most suitable technique for using the immobilized enzymes was to prepare each immobilized enzyme independently, so that the FIA system was constructed from 2 columns, an AOD–IE column and a POD–IE column, as shown in Figure 1.

**Injection Volume and Flow Rate**

The effect of sample volume was examined by injecting 0.1–1.5 mL of a 1 mg/dm³ ethanol solution. The maximum peak height was obtained for 1.3 mL.

The reaction period and the degree of sample dispersion in the immobilized enzyme column depended on the flow rate. To establish the optimum flow rate, the experiment was performed over a range of 0.4–1.4 mL/min for total flow rate of the double plunger pump by injecting a 1 mg/dm³ ethanol solution. The flow rates of the carrier and indicator solution

**Figure 5.** Effect of concentration of phosphate buffer in carrier stream on peak height. Indicator, 2.3 × 10³ mol/dm³ HPPA/0.3 mol/dm³ Tris; carrier, each concentration of phosphate buffer (pH 7); sample, 1.0 mg/dm³ ethanol (1.3 mL).

**Figure 6.** FIA signal for ethanol. Indicator, 2.3 × 10³ mol/dm³ HPPA/0.3 mol/dm³ Tris; carrier, 0.1 mol/dm³ phosphate buffer, pH 7; sample, each concentration of ethanol (1.3 mL).
Table 2. Effect of coexisting ion on the determination of ethanol

<table>
<thead>
<tr>
<th>Ion</th>
<th>Conc. of ion, mg/dm³</th>
<th>Relative activity of immobilized enzyme</th>
<th>Ion</th>
<th>Conc. of ion, mg/dm³</th>
<th>Relative activity of immobilized enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>10</td>
<td>98.4</td>
<td>Cl⁻</td>
<td>10</td>
<td>98.8</td>
</tr>
<tr>
<td>K⁺</td>
<td>10</td>
<td>97.7</td>
<td>Br⁻</td>
<td>10</td>
<td>99.7</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>10</td>
<td>97.5</td>
<td>I⁻</td>
<td>10</td>
<td>99.4</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>10</td>
<td>96.7</td>
<td>NO₃⁻</td>
<td>10</td>
<td>99.2</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>10</td>
<td>99.4</td>
<td>SO₄²⁻</td>
<td>10</td>
<td>98.5</td>
</tr>
<tr>
<td>EDTA²⁻</td>
<td>10</td>
<td>102.0</td>
<td>CO₃²⁻</td>
<td>10</td>
<td>96.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EDTA²⁻</td>
<td>10</td>
<td>97.5</td>
</tr>
</tbody>
</table>

* Added to 1.0 mg/dm³ ethanol.

Table 3. Effect of pretreatment with the ODS cartridge

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ethanol concn, mg/dm³</th>
<th>Recovery, %</th>
<th>Without pretreatment</th>
<th>With pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>10</td>
<td>100</td>
<td>105.1</td>
<td></td>
</tr>
<tr>
<td>Malic acid</td>
<td>10</td>
<td>96.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tartaric acid</td>
<td>10</td>
<td>100.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td>10</td>
<td>99.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malic acid</td>
<td>10</td>
<td>100.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinic acid</td>
<td>10</td>
<td>96.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malonic acid</td>
<td>10</td>
<td>99.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phthalic acid</td>
<td>10</td>
<td>97.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>10</td>
<td>105.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>10</td>
<td>97.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The effect of the concentration of phosphate buffer solution is shown in Figure 5. The buffer capacity was higher at higher concentrations of phosphate buffer in the carrier. Consequently, the pH of the reaction in the POD–IE column between HPPA and the hydrogen peroxide produced by the AOD–IE column was decreased. Therefore, the fluorescence generated by the dimerization of HPPA, which was catalyzed by POD–IE, was absorbed on the surface of the POD–IE so that the peak height was higher and the peak shape was tailing when a higher concentration of phosphate buffer was used as a carrier. On the basis of these results, the concentration of phosphate buffer in the carrier stream was set at 0.1 mol/dm³.

Effect of HPPA Concentration

HPPA was used as a fluorescence indicator in the proposed method; the reaction mechanism has been described in the literature (24). In our previous work (11), the sensitivity of the hydrogen peroxide determined by the FIA system, including the POD–IE column, was constant when the concentration of HPPA was >2.3 × 10⁻⁵ mol/dm³. In this study, the detection system was the same as that used previously (11); therefore, the HPPA concentration was set at 2.3 × 10⁻⁵ mol/dm³.

Stability of Immobilized Enzyme Column

In the proposed procedure the pH of the immobilized POD column was higher than that used in some other studies. After the experiments were performed, the POD column was filled with the preserving solution and stored in a refrigerator at 4°C. With this treatment, the immobilized POD column showed a constant sensitivity for >2 years. On the other hand, the immobilized AOD column was used without preservation. However, the activity of the immobilized AOD column decreased with increased use. In the FIA system, the sensitivity of the AOD column was constant for >2 weeks and >200 injections. Over this period, the sensitivity gradually decreased, but the concentration of ethanol determined from the calibration graph was corrected with the standard solution on the same day. The immobilized AOD column was used for the determination of ethanol in real samples for about 1 month, and it was
Figure 7. Results obtained by the method of standard additions for Japanese sake. Indicator, \(2.3 \times 10^3\) mol/dm\(^3\) HPPA/0.3 mol/dm\(^3\) Tris; carrier, 0.1 mol/dm\(^3\) phosphate buffer, pH 7; sample, ◦ is the calibration curve obtained with ethanol standard solution; ● shows the additions to Japanese sake, which was diluted to 1 to 50 000.

Table 4. Results for determination of ethanol in real samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>1/20 000 found</th>
<th>1/20 000 + 0.5 mg/dm(^3) ethanol</th>
<th>Recovery, %</th>
<th>Ethanol concn in real sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1</td>
<td>0.535</td>
<td>1.031</td>
<td>99.2</td>
<td>10.7</td>
</tr>
<tr>
<td>A-2</td>
<td>0.674</td>
<td>1.062</td>
<td>97.6</td>
<td>11.5</td>
</tr>
<tr>
<td>A-3</td>
<td>0.385</td>
<td>0.861</td>
<td>95.1</td>
<td>7.7</td>
</tr>
<tr>
<td>A-4</td>
<td>0.620</td>
<td>1.139</td>
<td>102.0</td>
<td>12.6</td>
</tr>
<tr>
<td>B</td>
<td>0.479</td>
<td>1.030</td>
<td>110.2</td>
<td>9.6</td>
</tr>
<tr>
<td>C</td>
<td>0.650</td>
<td>1.173</td>
<td>104.7</td>
<td>13.0</td>
</tr>
</tbody>
</table>

\(a\) A = Japanese sake; B = white wine; and C = sweet rice wine.
As pretreatment, the samples were filtered through a membrane filter (pore size, 0.45 μm) and then diluted 50 000-fold with water to within the linear range of the ethanol concentration for the proposed FIA method. Then the samples were injected through the ODS cartridge, for removal of organic interferences, into the FIA system shown in Figure 1. The regression equation obtained for the Japanese sake sample was parallel to the standard calibration graph that was obtained of the ethanol standard solution. On the basis of the results obtained by the method of standard additions, the proposed method was applied to the determination of ethanol in alcoholic beverages. Table 4 summarizes the concentrations found and results obtained after the addition of ethanol to the samples. The recovery results obtained by the proposed method were appropriate.

References