Immobilization of *Hansenula polymorpha* Alcohol Oxidase for Alcohol Biosensor Applications

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Alcohol oxidase catalyzes the oxidation of short lines alcohol to aldehyde. In this study, alcohol oxidase from *Hansenula polymorpha* (*Hp*AOD) was induced by addition of 0.5% methanol as the carbon source and purified to electrophoretic homogeneity by column chromatographies. The purified *Hp*AOD was immobilized with DEAE-cellulose particles and its biochemical properties were compared with those of free enzyme. The substrate specificity and the optimum pH of immobilized enzyme were similar to those of free enzyme. On the other hand, the K_m values of free and immobilized enzymes for ethanol were 6.66 and 14.65 mM, respectively. The optimum temperature for free enzyme was 50°C, whereas that for immobilized enzyme was 65°C. Immobilized enzyme showed high stability against long storage. Immobilized enzyme was also tested for the enzymatic determination of ethanol by the colorimetric method. We detected 1 mg/liter ethanol (1×10^{-4}% ethanol) by 2,6- dichloroindophenol system. Therefore, the present study demonstrated that immobilized *Hp*AOD has high substrate specificity toward ethanol and storage stability, which may be of considerable interest for alcohol biosensor and industrial application.

Key Words: *Hansenula polymorpha*, Alcohol oxidase, Biochemical properties, Immobilization, Stability

Introduction

Alcohol oxidase (alcohol: oxygen oxidoreductase, EC 1.1.3.13, AOD) is an oligomeric enzyme with eight identical subunits, each containing a non-covalently bound flavine adenine dinucleotide molecule (FAD) as a cofactor.[1,2] AOD catalyzes the oxidation of primary low molecular weight alcohols into the corresponding aldehydes. During this reaction, cofactor (FAD) in AOD is first reduced to its hydrogenated form ($FADH_2$) and then re-oxidized to its native form by molecular oxygen (O_2), with the concomitant formation of hydrogen peroxide (H_2O_2).

One of the most relevant applications of AOD has been the monitoring of ethanol in the beverages and fermentation industries, clinical chemistry and forensic analysis.[3] The accurate quantification of ethanol with high sensitivity and selectivity in biological fluids such as plasma and urine and in human breath is required for clinical and forensic laboratories. The industries such as production of alcoholic beverages, food-stuffs, cosmetic and pharmaceuticals are very interested in fast analytical methods to control fermentation process and product quality. A variety of methods and strategies for the analysis of ethanol in complex samples have been reported including liquid and gas chromatography, refractometry and spectrophotometry.[4,5] On contrary to conventional methods, the application of enzymes allows to considerable enhancement of specificity and thus decreases the expenditure for sample pretreatment.[6]

AOD has also attracted major scientific interest for use in the bioconversion of ethanol to acetaldehyde. Acetaldehyde is an important bulk chemical and is mostly used for purposes of acetic acid synthesis.[7] Moreover, acetaldehyde is used as a fragrance and flavor additive by the food industry.[8] Some bioconversion processes have been described utilizing alcohol dehydrogenases, either purified or in tact cells, for the conversion.[9] However, alcohol dehydrogenase requires a cofactor, NADH, which needs continuous regeneration. As an alternative, AOD does not require cofactor regeneration.[10] Bioconversion of ethanol to acetaldehyde has been reported using either purified AOD or intact AOD-containing cells from methylotrophic yeasts.[8,9,11,12]

In these respect, immobilized AOD is advantageous over the native enzyme because of its thermal resistance and tolerance towards the common denaturing reagents, and it is preferred over the native ones owing to their multiple and repetitive use. Furthermore, immobilized enzyme has a longer life time and predictable decay rate.[13-17]

In this study, alcohol oxidase from *Hansenula polymorpha* was induced, purified and immobilized with DEAE-cellulose particles. The biochemical properties of immobilized enzyme were compared with those of free enzyme. We also described the colorimetric method for ethanol determination by immobilized enzyme.

Materials and Methods

Materials. *Hansenula polymorpha* (A.T.C.C. 26012) was obtained from Korean Culture Center of Microorganisms in freeze-dried form. Yeast nitrogen base without amino acid, yeast extract and bactopeptone were obtained from Difco Laboratories (Detroit, USA). 2,6-Dichloroindophenol (DCIP), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), leticene (zymolyase) and peroxidase were purchased from Sigma (St Louis, MO, USA). DEAE-Sephacel and Mono Q HR 5/5 were obtained from Pharmacia Biotech (Uppsala, Sweden). Protein-assay reagent and SDS-PAGE molecular mass markers were purchased from Bio-Rad (Hercules, CA, USA). All reagents used were of the highest grade available commercially.

Expression and purification of alcohol oxidase from

Hansenula polymorpha. Cells of the methylotrophic yeast *Hansenula polymorpha* were grown with shaking in YNB medium (0.7% yeast nitrogen base without amino acid, 0.1% yeast extract and 0.05% bacto peptone) at 37°C for 16-24 hours. Alcohol oxidase was strongly induced by addition of 0.5% methanol as the carbon source. The cells were harvested by centrifugation at 10,000 g for 10 min and then were suspended in 50 mM tris-HCl buffer contained 10 mM $MgCl_2$, 1 M sorbitol and 30 mM dithiothreitol, pH 7.5. The cells were subjected to the addition of zymolyase (200 U) for 40 minutes and to sonication using an ultrasonic processor (Sonics and Materials, Danbury, CT, USA) until lysed. The disrupted cell suspension was centrifuged at 40,000 g for 60 min, yielding the crude extracts. The supernatant was dialyzed against 50 mM potassium phosphate buffer, pH 7.5 (buffer A) and loaded on to a DEAE-Sephacel column (1.6 × 40 cm) previously equilibrated in buffer A. After washing the column with buffer A, bound proteins were eluted with a linear gradient of 0-1 M NaCl in buffer A at a flow rate of 0.25 ml/min. Fractions containing enzyme activity were pooled, dialyzed against buffer A, and loaded on to a Mono Q HR 5/5 column equilibrated in buffer A. The column was washed with buffer A and bound proteins were eluted with a linear gradient of 0-500 mM NaCl in buffer A at a flow rate of 0.5 ml/min. Active fractions were pooled and were dialyzed against buffer A. The purified enzyme was essentially homogeneous as judged by SDS-PAGE (data not shown) and the specific activity of the enzyme toward ethanol was approximately 10 Unit/mg. This purified enzyme was used for an immobilization experiment. Unless otherwise indicated, all purification procedures were performed either at 4°C or on ice. The enzymes were stored at -70°C until use.

Activation of DEAE-cellulose with cyanuric chloride. The DEAE-cellulose was activated by slightly modified method proposed by Tiller *et al*.[18] The pre-swollen DEAE-cellulose was washed and suspended with distilled. The suspended solution was saturated by sodium hydroxide. The remaining sodium hydroxide in the solution was removed by filtration. The DEAE-cellulose was added to a solution of 100 mg of cyanuric chloride dissolved in 10 ml of 1:1 (w/w) dioxane-xylene solution and incubated at room temperature for 30 min. The activated surface was then thoroughly rinsed with ethanol until it became clear, washed with 50 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.5, and immediately placed into an enzyme solution in the same buffer, which had previously been cooled to 4°C.

Enzyme immobilization. The purified enzyme (300 Units) was mixed with the activated cellulose support (1 g). After incubation at 4°C for 24 hour, the nonattached enzyme was removed by rinsing with 1 M NaCl. The supernatant was taken to determine the concentration of free enzymes, and the enzyme immobilized-cellulose was used for the assay after washing several times with buffer. For each experiment, three replicates were studied and an average of the three was taken as the result.

Enzyme assay and kinetic studies. Alcohol oxidase-catalysed reactions were routinely performed spectrophotometrically at 405 nm in coupled assays with 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and peroxidase as described by Tani *et al*. (1985).[19] Molar absorbance coefficient of ABTS was 36,800 $M^{-1}\cdot cm^{-1}$ and one unit of enzyme activity was defined as the amount of enzyme that catalyzed the generation of 1 μmole of H_2O_2 per min under the above experimental conditions. Specific activity was expressed as units per milligram of protein. Kinetic parameters were determined by Lineweaver-Burk plot method.[20] The enzyme concentration used for initial-rate studies was 1 ~ 50 μg/ml and the concentration of ethanol was varied over the range 0.1 ~ 100 mM.[21] The protein concentration of the enzyme was determined by the method of Bradford using γ-globulin as standard.[22]

Enzyme stability. The stabilities of the enzymes were determined by using native and immobilized enzymes with a protein concentration of 0.1 mg/ml. The enzymes were incubated in buffer A at 4 °C or 25 °C over 7 weeks. At the end of the storage period, the remaining activity was determined.

Colorimetric method of the enzymatic analysis for determination of ethanol. For the direct determination of ethanol using the enzyme, 1.5 ml of 10 mM potassium phosphate buffer (pH 7.5) containing specific amounts of immobilized AOD, peroxidase of 4 units/ml and 100 mM 2,6-dichloroindophenol (DCIP) was mixed with 0.5 ml of an ethanol solution (in the same buffer) of known concentration and incubated at 25°C. DCIP was well soluble in water and hence could be uniformly dispersed during the preparation of the preparation of the enzyme-support power. The absorbance spectrum in the visible range for an aqueous solution of this compound in its oxidized form had a maximum at 610 nm. Consequently, the reaction was monitored by measuring changes in absorbance at this wavelength using a spectrophotometer.

Results and Discussion

Catalytic properties and stabilities of free and immobilized enzymes. The immobilization of *Hp*AOD was performed with the activated cellulose support as mentioned in the Materials and Methods. The substrate specificity of immobilized enzyme towards several compounds was shown in Table 1. A number of primary, secondary and tertiary alcohols were used to determine the substrate specificity of immobilized enzyme. A gradual decrease in rate was observed by branching of the carbon chain of the alcohol. Immobilized enzyme oxidized mainly primary aliphatic alcohols and exhibited high substrate specificity towards ethanol and methanol. On the other hand, immobilized enzyme didn't show activity towards long chain primary alcohols, short chain secondary alcohols and tertiary alcohols. This substrate specificity of immobilized enzyme was similar to free enzyme, although immobilized enzyme showed approximately 20-30% lower activities than those of free enzyme.

The kinetic parameters of free and immobilized enzymes were shown in Table 2. The maximum reaction rate (V_{max}) describes the rate of reaction between enzyme and its substrate, ethanol and Michaelis-Menten constant (K_m) defines the affinity of enzyme toward ethanol. The V_{max} value of immobilized enzyme for ethanol was 15.38 U/mg. This value is similar to

Table 1. Substrate specificity of free *Hp*AOD and immobilized *Hp*AOD

Substrates	Free *Hp*AOD		Immobilized *Hp*AOD	
	Specific Activity (U/mg)	Relative Activity (%)	Specific Activity (U/mg)	Relative Activity (%)
1-butanol	0.04 ± 0.002	0.4	ND[a]	-
2-butanol	ND[a]	-	ND[a]	-
3-butanol	ND[a]	-	ND[a]	-
crotyl alcohol	0.68 ± 0.03	6.6	0.48 ± 0.01	5.9
ethanol	10.23 ± 0.51	100	8.18 ± 0.43	100
isoamyl alcohol	0.02 ± 0.001	0.2	ND[a]	-
methanol	9.48 ± 0.32	92.7	6.68 ± 0.62	81.7
1-propanol	0.21 ± 0.02	2.1	0.33 ± 0.004	4.0
2-propanol	0.01 ± 0.001	0.1	ND[a]	-
2,2,2-trichloroethanol	0.02 ± 0.002	0.2	ND[a]	-

[a]ND, Not detected. The values showed are Means ± S.D., generally based of n ≥ 5.

Table 2. Kinetic parameters for free *Hp*AOD and immobilized *Hp*AOD

	Free *Hp*AOD	Immobilized *Hp*AOD
K_m (mM)	6.66	14.65
V_{max} (U/mg)	17.24	15.38

The values showed are means, generally based of n ≥ 3.

that of free enzyme (17.24 U/mg). The K_m values of free and immobilized enzymes for ethanol were determined as 6.66 mM and 14.65 mM, respectively. Higher the K_m value means lower its affinity against its substrate. Thus, immobilized enzyme has a weaker affinity toward ethanol than free enzyme. From these results on the substrate specificity and the kinetic parameters, we suggest that immobilization of *Hp*AOD did not largely affect the correct orientation between the active site of enzyme and substrate, although it may more or less affect substrate binding in enzyme-substrate complex.

The biochemical properties of immobilized enzyme were investigated with ethanol oxidation reaction. The effects of pH on ethanol oxidation by free and immobilized enzymes were shown in Figure 2. The optimum activity of immobilized enzyme on ethanol was observed in pH 8.5. Immobilized enzyme showed less than 45% of its maximum activity below pH 4.0 and appeared approximately 85% of its maximum activity even at pH 10.0. Similar result was also found in free enzyme.

Immobilized enzyme was shown to be active over a wide range of temperatures (from 30 to 80°C). The optimum temperature of immobilized enzyme was 65°C and up to 25% of activity was observed even at 80°C. On the other hand, the optimum temperature of free enzyme was 50°C. The thermostability of immobilized enzyme was investigated by incubation of the enzyme at various temperatures for 10 min. The midpoints of the temperature-stability curve for free and immobilized enzymes were approximately 54°C and 63°C, respectively. Immobilized enzyme was fairly stable at temperature up to 60°C. Above 70°C, its activities declined rapidly as the temperature increased, but immobilized enzyme was not completely inactivated even at 80°C. Therefore, immobilization of *Hp*AOD with the activated cellulose support supplies a higher stability.

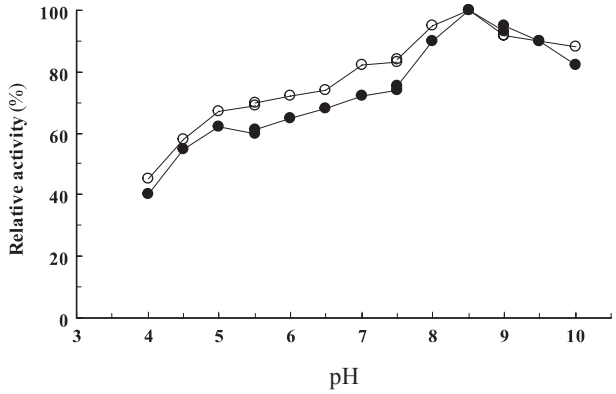

Figure 2. Effect of pH on free *Hp*AOD and immobilized *Hp*AOD. The buffer conditions as follow: pH 3.5-5.5, 100 mM sodium acetate buffer; pH 5.5-7.5, 100 mM potassium phosphate buffer; pH 7.5-9.0, 100 mM Tris-HCl buffer; pH 9.0-10.0, 100 mM sodium carbonate-bicarbonate buffer. The maximum activity was expressed 100%. Indicate that free *Hp*AOD (-●-) and immobilized *Hp*AOD (-○-).

Enzymes can lose their catalytic activity and be denaturated easily. Hence, storage stability is a very important factor for the practical use of enzyme in biosensors or as biocatalysts. Immobilized enzyme showed a high storage stability and retained approximately 90% of its original activity even after

Figure 1. Schematic diagram for the activation of cellulose by cyanuric chloride and immobilization of *Hp*AOD by covalent linkage.

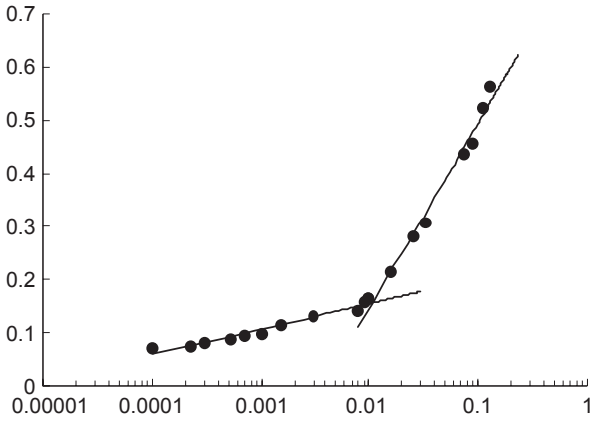

Figure 3. Determination of ethanol concentration using immobilized *Hp*AOD with DCIP system. The concentration of DCIP is proportional to the original ethanol concentration and can be deter- mined by its absorbance at 610 nm.

10 weeks at 4°C. On the other hand, free enzyme retained 70% of its original. At 25°C, immobilized enzyme retained approximately 60% of its original activity after 10 weeks. On the other hand, free enzyme retained only 20% of its original activity. These results indicate that immobilized enzyme has very good stability at 4°C and can be safely used in 10 weeks. This stable immobilized *Hp*AOD may be useful for the enzymatic determination of alcohol and for the industrial production of alcohols and aldehydes.

The determination of ethanol concentration using immobilized enzyme. Alcohol oxidase catalyzes the oxidation of alcohols to aldehydes and has been used extensively for the determination of ethanol in body fluids.[23,24] We performed by evaluating a chromogenic agent, 2,6-dichloroindophenol (DCIP) for its ability to develop a stable and distinct change in color when subjected to peroxidase-catalyzed oxidations. The reaction scheme is as follows:

$$CH_3CH_2OH + O_2 \longrightarrow CH_3CHO + H_2O_2$$

$$DCIP_{oxidized} + H_2O_2 \longrightarrow DCIP_{decomposed} + H_2O$$

The enzymatic system composed of immobilized *Hp*AOD/peroxidase/$DCIP_{oxidized}$ was characterized in aqueous solution, and the results obtained were shown in Figure 3. As the response to ethanol in the range 1-1,500 mg/liter ethanol (1×10^{-4} − 1.5×10^{-1} %) was not linear, the calibration curves were transformed the values of ethanol concentration by log to obtain the linear dependencies. Similar behavior of ethanol biosensors has been described.[24] Under the experimental conditions the lowest detectable ethanol concentration was 1 mg/liter ethanol (1×10^{-4} %) and the calibration curve was linear up to concentrations of 100 mg/liter ethanol (1×10^{-2} %). Moreover, the procedure was very rapid showing the detection time of 1 min. The calibration curve was also linear in the range 100-1,500 mg/liter ethanol (1×10^{-2} − 1.5×10^{-1} %). The detection limit of this colorimetric method was significantly lower than those of other methods for alcohol determination,[25,26] and it was sufficient to monitor ethanol in clinical applications with reference to urine or blood.

In conclusion, we purified an AOD from *H. polymorpha* and immobilized the purified enzyme with the activated cellulose support. The substrate specificity, the kinetic parameters and the optimum pH of immobilized enzyme were similar to those of free enzyme, although distinct features can be pointed out, as mentioned above. Particularly, immobilized enzyme has a higher stability and it was useful for the enzymatic determination of alcohol with DCIP system.

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