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Purification and Properties of a Membrane-bound Alcohol Dehydrogenase from *Acetobacter* sp. HA

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Membrane-bound alcohol dehydrogenase (ADH) was purified to homogeneous state from an acetic acid producing bacteria, *Acetobacter* sp. HA. The enzyme was purified about 153-fold with an overall yield of 35% from the crude cell extract by solubilization and extraction of the enzyme with Triton X-100 and subsequent fractionations by column chromatography. Upon sodium dodecyl sulphate-PAGE, the enzyme showed the presence of three subunits with a molecular mass of 79,000 daltons, 49,000, and 45,000 daltons, respectively. Absorption spectra showed the presence of cytochrome c in the purified ADH. The ADH preferentially oxidized aliphatic alcohols with a straight carbon chain except for methanol. Formaldehyde, acetaldehyde and glutaraldehyde were also oxidizable substrates. The apparent K_m for ethanol was 1.38 mM. The optimun pH and temperature were 5.0~6.0 and 32°C, respectively. V_2O_5 and heavy metals such as $ZnCl_2$ and $NiCl_2$ were inhibitory to the enzyme activity.

KEY WORDS *Acetobacter* **sp. HA, alcohol dehydrogenase, purification, characterization**

Acetic acid fermentation is the process whereby acetic acid is produced from ethanol by acetic acid bacteria. Based on their physiological characteristics in carbohydrate metabolism, acetic acid bacteria are classified into two genera, *Acetobacter* and *Gluconobacter*. Both of them have strong ability to oxidize sugars and alcohols (6, 12).

Alcohol dehydrogenase (ADH) of acetic acid bacteria acts on a wide range of primary alcohols except for methanol. The enzyme is involved in oxidation of ethanol to acetic acid via acetaldehyde by coupling with aldehyde dehydrogenase (ALDH). The enzyme is localized on the outer surface of cytoplasmic membrane, and the oxidation of substrates is linked to its respiratory chain (3). The enzyme differs from alcohol dehydrogenase (EC 1.1.99.8) of methanol utilizing bacteria, which, without exception, requires ammonia for full activity (4).

To elucidate the action mechanism of ADH and the acid tolerance of the acetic acid bacteria, extensive purification and characterization of the enzymes involved in ethanol oxidation of *Acetobacter aceti*, *Acetobacter rancens*, *Acetobacter polyoxygens* and *Gluconobacter suboxydans* have been performed (1, 2, 9, 14).

Recently, a strain of acidophilic and acetogenic bacterium, *Acetobacter* sp. HA was isolated by selective enrichment from the fermented rice wine vinegar in Korea and was identified as a new strain (5). As a first step to elucidate the action mechanism of ADH and the acid tolerance of this strain, alcohol dehydrogenase was purified to near homogeneity.

This paper describes purification and some catalytic and physicochemical properties of the ADH of *Acetobacter* sp. HA.

MATERIALS AND METHODS

Microorganisms and cultivation

Bacterial strains used in this study was *Acetobacter* sp. HA which was isolated from the traditional raw rice wine vinegar produced in the Haenam area (5). Culture medium employed in this study contained 5 g of yeast extract, 3 g of peptone, 5 g of D-glucose and 10 m*l* of ethyl-alcohol in 1 liter of distilled water. *Acetobacter* sp. HA was inoculated into 100 m*l* of the medium in a 500 m*l* shaking flask, and the cultivation was carried out at 27°C for 24 hour with reciprocal shaking (150 rpm).

Enzyme assay

The ADH activities were assyed by the methods of Ameyama and Adachi (3). One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmole ethanol per

min. The enzyme activity was measured at pH 6.0 at 32°C unless otherwise stated. The effect of pH on the enzyme activity was examined under the conditions described above except for the use of McIlvaine buffer (pH 2.5~9.0).

Protein determination

Protein content was determined according to the method of Lowry *et al.* (10), using bovine serum albumin (Sigma) as a standard.

Purification of alcohol dehydrogenase

All the procedures for purification of the enzyme were done at 4°C. Washed cells (wet weight, 6.0 g) were suspended in 20 m*l* of 0.01 M of potassium phosphate buffer (pH 6.0) and disrupted by sonication. The suspension of disrupted cells (crude-cell extract) was centrifuged at 120,000×*g* for 1 hour. The pellet was used as the membrane fraction.

The membrane fraction was suspended in 20 m*l* of buffer A [0.01 M potassium phosphate buffer (pH 6.0) containing 0.1% (W/V) Triton X-100]. The mixture was stirred for 2 hours and then centrifuged at 70,000×*g* for 1 hour. The supernatant was used as the solubilized fraction.

The solubilized fraction was then applied to a DEAE-Sephadex A-50 (Sigma) column (1.2×25 cm) previously equilibrated with buffer A. The column was washed with 50 m*l* of buffer A and then eluted with linear phosphate gradient (0.01 M~0.05 M). The active fractions were collected and dialyzed against 0.005 M phosphate buffer (pH 6.0) containing 1% Triton X-100.

The dialyzed active fraction was loaded onto a hydroxyapatite (Bio-Rad) column (1.5×4 cm) previously equilibrated with 0.005 M phosphate buffer (pH 6.0) containing 1% Triton X-100. The column was washed with 50 m*l* of 0.1 M phosphate buffer (pH 6.0) containing 1% Triton X-100 and then eluted with a linear phosphate gradiant (0.1 M~0.2 M). The active fractions obtained from the hydroxyapatite column were concentrated and dialyzed against 0.05 M phosphate buffer (pH 6.0) containing 1% Triton X-100.

Table 1. *Purification of alcohol dehydrogenase from Acetobacter sp. HA.*

Purification steps	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude cell extract	75	88	0.85	100
Solubilized fraction	72	21	3.40	96
DEAE-Sephadex A-50	43	4.9	10.40	57
Hydroxyapatite	26	0.2	130.0	35

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate (SDS)-PAGE were performed by the methods described by Adachi *et al.* (1) using 7.5% acrylamide gel and 10% acrylamide gel containing 0.1% (W/V) SDS, respectively. The molecular weight markers obtained from Gibco BRL were myosin (200,000 daltons), rabbit muscle phosphorylase b (97,400 daltons), bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (29,000 daltons), and lysozyme (14,300 daltons).

Gel filtration

For the estimation of the molecular weight, the purified enzyme was applied to a Sephadex G-200 column (1.45×74 cm) and eluted with 0.05 M potassium phosphate buffer (pH 6.0) containing 0.1% (W/V) Triton X-100. Standard proteins (Sigma) used were ferritin (440,000 daltons), yeast alcohol dehydrogenase (150,000 daltons), bovine serum albumin (66,000 daltons) and carbonic anhydrase (29,000 daltons)

RESULTS AND DISCUSSION

Purification of ADH from *Acetobacter* sp. HA

The results of enzyme purification are summarized in Table 1. Almost of the enzyme activity was recovered in the solubilized fraction. This indicated that most of the enzyme was localized in the cytoplasmic membrane of this

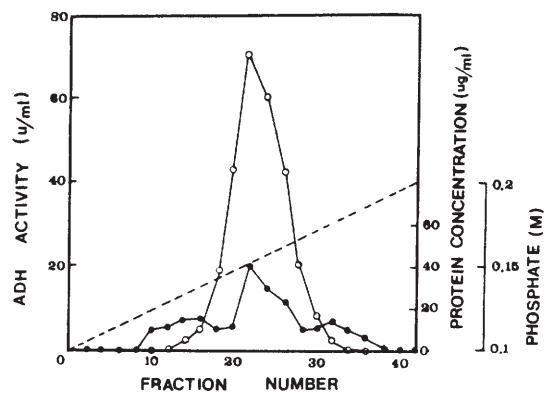

Fig. 1. *Hydroxyapatite column chromatography of alcohol dehydrogenase from Acetobacter sp. HA.*

The enzyme solution obtained from DEAE-Sephadex A-50 was applied to a hydroxyapatite column (1.5×4 cm) previously equilibrated with phosphate buffer (2 mM, 0.1 % Triton X-100). The enzyme was eluted with a linear phosphate gradient (0.1~0.2 M) at a flow rate 6 ml/h. (○-○, ADH activity; ●-●, protein conc.; ----, phosphate).

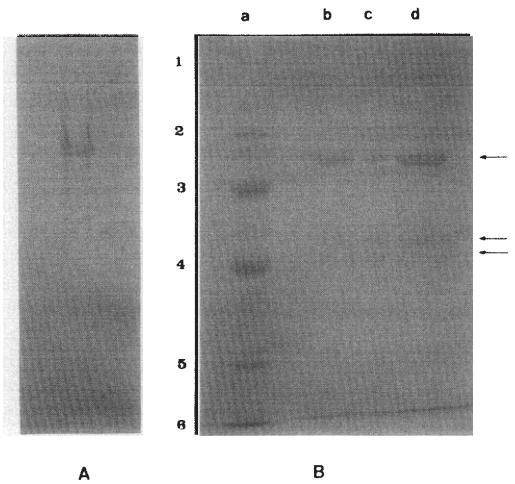

Fig. 2. *Polyacrylamide gel (7.5%) and SDS-poly acrylamide gel (10%) electrophoresis of purified alcohol dehydrogenase from Acetobacter sp. HA. (A, PAGE; B, SDS-PAGE).*
a, Size marker: 1, myosin (200,000 Da); 2, rabbit muscle phosphorylase b (97,400 Da), 3, bovine serum albumin (68,000 Da); 4, ovalbumin (43,000 Da), 5, carbonic anhydrase (29,000 Da); 6, lysozyme (14,300 Da). b, purified enzyme 10 μg; c, 5 μg; d, 20 μg.

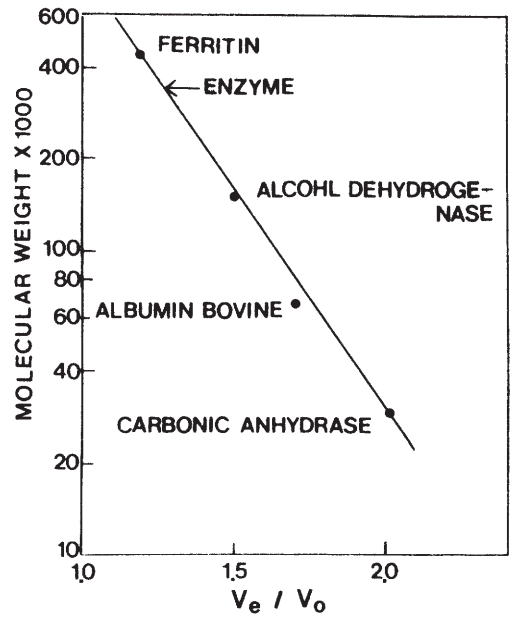

Fig. 3. *Determination of the molecular weight of alcohol dehydrogenase from Acetobacter sp. HA by Sephadex G-200 gel filtration.*
arrow, ADH of *Acetobacter* sp. HA.

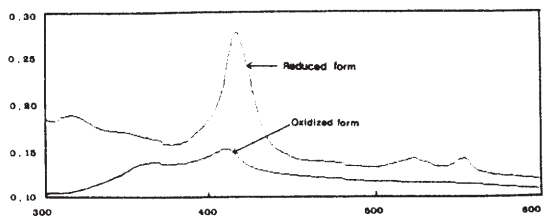

Fig. 4. *Absorption spectra of alcohol dehydrogenase from Acetobacter sp. HA.*
Purified enzyme solution with a specific activity of 130 (U/mg) was used. Oxidized form of the enzyme was prepared by incubating the enzyme at pH 12 for 1 hr in the presence of 0.1% Triton X-100.

strain. When ADH was eluted from the hydroxyapatite column, the enzyme activity was observed at around 0.15 M phosphate as shown in Fig. 1. Polyacrylamide gel electrophoresis of the enzyme solution obtained from hydroxyapatite column chromatography showed a single band of protein as shown in Fig. 2-A. The overall process achieved about 153 fold purification with a yield of about 35%.

Physicochemical properties of the ADH

The molecular weight of the purified enzyme was estimated to be 330,000 daltons by gel filtration with Sephadex G-200 (Fig. 3). The subunit structure was analyzed by SDS-PAGE and the results are shown in Fig. 2-B, which indicates three bands of stained proteins. The molecular weight of each subunit was about 79,000 daltons, 49,000 daltons and 45,000 daltons, respectively. Considering the molecular weight of the native enzyme and band intensities of subunits on SDS-PAGE, it appeared that the holoenzyme was composed of three molecules of the 79,000 daltons subunit, one molecule of 49,000 daltons subunit and one molecule of 45,000 daltons subunit. It was reported that the molecular weight of the ADH from *A. polyoxygens* was 320,000 daltons and consisted of two subunits, each having 72,000 and 44,000 daltons, respectively (14). Compared with the ADH from *A. polyoxygens*, the holoenzyme of *Acetobacter* sp. HA had similar molecular weight, although numbers and molecular weight of subunits were different from each other. Also, differences were observed compared with the enzyme from *A. aceti* (14), which had four subunits, each having 63,000, 44,000, 29,000 and 13,500 daltons.

The purified enzyme was rose-red colored and showed a cytochrome c-like absorption spectrum as shown in Fig. 4. The maximum absorption at 418, 523, and 553 nm were observed with the reduced enzyme and a sole peak at 409 nm was

Table 2. *Substrate specificity of alcohol dehydrogenase from Acetobacter sp. HA.*

Substrate	Relative activity(%)	Substrate	Relative activity(%)
Ethanol	100	tert-Butanol	0
Methanol	0	n-Butanol	100
Gluconate	0	n-Pentanol	90
Glucose	0	n-Hexanol	75
Mannitol	0	1-Octanol	41
Sorbitol	0	Formaldehyde	32
Lactic acid	0	Glutaraldehyde	25
Glycerol	0	Acetaldehyde	25
Fructose	0	Isopropanol	19

Each substrate was added at a final concentration of 100 mM.

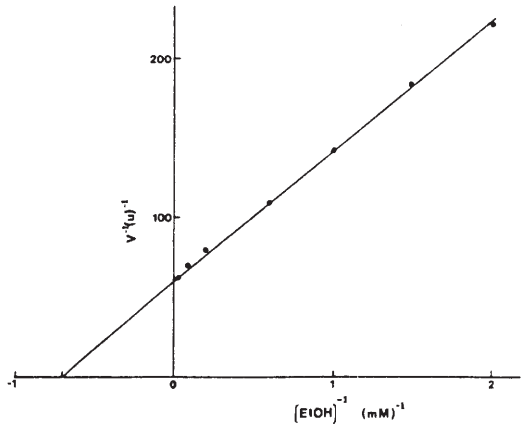

Fig. 5. *Lineweaver-Burk plot of alcohol dehydrogenase from Acetobacter sp. HA reaction rate against the ethanol concentration.*

observed with the oxidized form, which coincided with those of cytochrome c (1). These absorption spectra were also quite similar to those from *A. polyoxygens* and *A. aceti* (2, 14). This result indicated that ADH from *Acetobacter* sp. HA contained cytochrome c, which has a function in ethanol oxidation of acetic acid bacteria (14).

Catalytic properties

The relative activities of the purified ADH toward various alcohols, aldehydes and other organic compounds were shown in Table 2.

The enzyme showed strong activity toward aliphatic alcohols with straight carbon chains excepts for methanol. Among the branched chain and aromatic alcohols examined, isopropanol was weakly oxidized but tert-butanol and aromatic alcohols were not oxidized to an appreciable extent. Formaldehyde, acetaldehyde and glutaraldehyde were also oxidized. It was reported

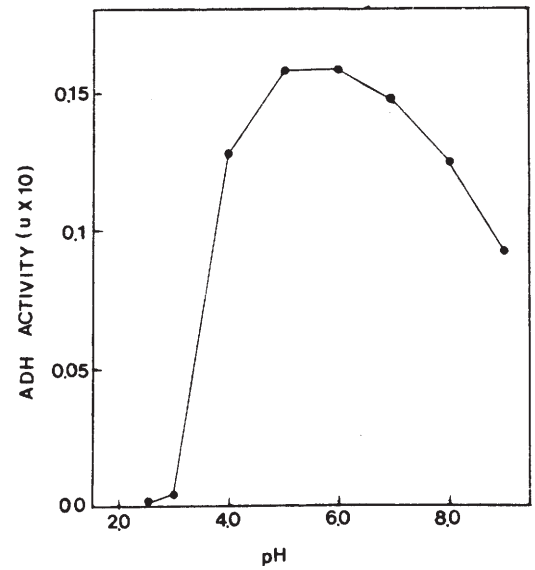

Fig. 6. *Effect of pH on alcohol dehydrogenase activity in McIlvaine buffer (pH 2.5~9.0).*

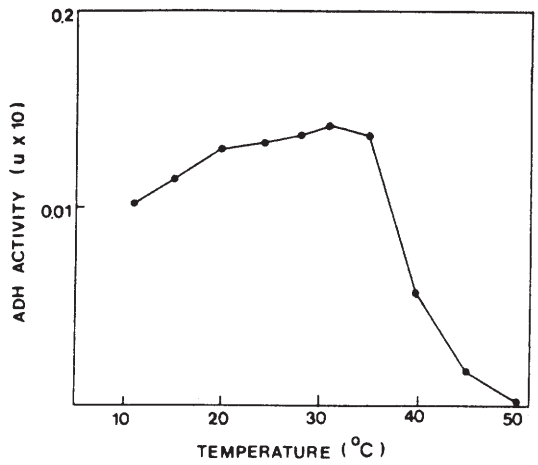

Fig. 7. *Effect of temperature on alcohol dehydrogenase activity.*

that aliphatic alcohols with a straight carbon chain were oxidized by ADHs from *A. aceti*, *A. suboxydans* and *A. polyoxygens*, but formaldehyde and acetaldehyde were oxidized only by ADH of *A. polyoxygens* (1, 2, 14). So, profile of substrate specificity of this enzyme was quite similar to that from *Acetobacter polyoxygens* sp. nov. The Michaelis constant (K_m) for ethanol was 1.38 mM (Fig. 5). Optimum pH of ethanol oxidation was shown in Fig. 6, and the enzyme showed its maximum activity at pH 5.0 to 6.0. Apart from

Table 3. *Effects of various compounds on alcohol dehydrogenase activity from Acetobacter sp. HA.*

Compounds (1 mM)	Remaining activity (%)
None	100
Sodium azaid	100
Sodium arsenate[a]	96
V_2O_5	50
EDTA[b]	100
$CuSO_4$	81
$ZnCl_2$	14
$NiCl_2$	61
$MnCl_2$	77
$MgCl_2$	98
$CoCl_2$	100

The enzyme was incubated with the compound for 10 min before measuring activity.
[a]Sodium arsenate was added at a final concentration of 10 mM.
[b]Ethylenediaminetetraacetate.

this pH region, enzyme activity was gradually decreased. There was a marked difference in pH optima when the enzyme was compared with those of primary alcohol dehydrogenase of methanol utilizing bacteria which show their maximal reaction rate at fairly alkaline pH (11, 13, 15).

The optimum temperature was 32°C and, above 40°C, enzyme activity decreased sharply (Fig. 7). The effects of inhibitors on enzyme activity were also examined. As shown in Table 3, $ZnCl_2$ and V_2O_5 inhibited the activity markedly. $NiCl_2$, $MnCl_2$ and $CuSO_4$ were also inhibitory, but EDTA did not affect the activity.

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초 록: *Acetobacter* sp. HA로부터 Membrane-bound Alcohol Dehydrogenase의 정제 및 특성

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초산 생성 균주인 *Acetobacter* sp. HA로부터 membrane-bound alcohol dehydrogenase(ADH)를 분리 정제하였다. 세포막 분획과 세포막에서 효소의 용출 그리고 크로마토그라피 방법 등을 적용하여 수율 35%, 153배 정제된 효소를 획득하였다. 정제된 효소의 분자량은 330,000 dalton이었으며, 각각 분자량 79,000과 49,000 그리고 45,000 dalton을 가진 세 종류의 subunit로 이루어져 있었다. 그리고 흡수 스펙트럼의 분석 결과 본 효소에는 cytochrome c가 존재함을 확인할 수 있었다. 본 효소는 메탄올을 제외한 1차 지방족 알코올을 기질로 이용할 수 있었으며, formaldehyde, acetaldehyde 그리고 glutaraldehyde의 경우에도 다소간 기질로 이용될 수 있었다. 본 효소의 에탄올에 대한 K_m값은 1.38 mM이었으며, 최적 pH와 온도는 각각 5.0~6.0과 32°C이었다. 본 효소의 활성은 V_2O_5와 $ZnCl_2$, $NiCl_2$같은 금속 이온에 의하여 저해되었다.