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Characterization of Alcohol Dehydrogenase Encoded by *Zymomonas mobilis* Gene Cloned in *Escherichia coli*

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Escherichia coli 형질전환체가 생산하는 *Zymomonas mobilis* 알콜 탈수소 효소의 분석

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The structural gene (*zadh*II) encoding an alcohol dehydrogenase II from *Zymomonas mobilis* was cloned into *Escherichia coli* in our laboratory (Yoon *et al.*, 1989. *Kor. J. Microbiol. Biotechnol.*). From *E. coli* (pADS93) carrying the *zadh*II gene, the *Z. mobilis* alcochol dehydrogenase II (ZADH-II) was purified by sonication, $(NH_4)_2 SO_4$ fractionation, and chromatography. The ZADH-II enzyme produced by *Z. mobilis* cell was also purified to compare to the enzyme produced by *E. coli* (pADS93). The purified enzyme from cell extract of *E. coli* (pADS93) was identified to be a tetramer being composed of four identical subunits having molecular weight of 40,000 dalton like that of *Z. mobilis*. The pH optimum for the reaction oxidizing ethanol to acetaldehyde was 10.0 while the optimum for the reverse reaction was 7.5-8.5. The apparent K_m values for ethanol and NAD+ were 1.2×10^{-1} M and 5.1×10^{-5} M, respectively. In addition, it was found that the K_m value for acetaldehyde was very lower than that for ethanol.

Alcohol dehydrogenase (ADH), which catalyzes the interconversion of alcohol and aldehyde in the presence of NAD(P)+ as a cofactor, is widely distributed through nature and has been observed in various organisms including bacteria, yeasts, fungi, insects, plants and mammalian cells. ADH responsible for the final step during alcoholic fermentation was purified from *Z. mobilis* by several groups (1-3). There are two different forms of ADH in *Z. mobilis* cells. The isozymes with faster electrophoretic mobility (ZADH-II) was identified to be an iron-activated enzyme. Recently, the structural gene (*adhB* = *zadh*II) encoding *Z. mobilis* ADH-II has been cloned and sequenced (4-6). On the basis of the nucleotide sequence, the genes of *Z. mobilis* ATCC 10988 and strain ZM4 coincide except 13 positions (6). In this study, the ZADH-II produced by *E. coli* transformant was purified and characterized.

Key words: Alcohol dehydrogenase, purification, characterization, *Z. mobilis*, *E. coli* (pADS93))
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Materials and Methods

Bacterial strains and growth conditions

E. coli (pADS93), which was derived by transforming *E. coli* JM83 with plasmid pADS93 containing the *zadh*II gene of *Z. mobilis* ATCC 10988, was used to produce the enzyme ZADH-II. The enzyme was also prepared from *Z. mobilis* ATCC 10988. In the LB medium (1.0% tryptone, 0.5% yeast extract and 0.5% NaCl) supplemented with ampicillin (50 μg/m*l*), *E. coli* (pADS93) was grown for 16 h at 37°C with vigorous shaking. *Z. mobilis* cells were cultured in RM broth consisting of 20g glucose, 10g yeast extract and

2g KH_2PO_4 per liter, pH 5.5 without shaking at 30°C.

Enzyme purification with cell extract of *E. coli* (pADS93)

All steps for enzyme purification were performed at 4°C. Approximately 4g wet weight of *E. coli* (pADS93) cells was washed with 30 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM cobaltous chloride, and suspended in a minimal volume of the same buffer. The cell suspension was sonicated for 10 min with Branson Sonifier Model 350 at 40% output. Cell debris was removed from the extract by centrifugation (20 min, 15,000 × g). Solid ammonium sulphate was added to the supernatant to be the final concentration of 25%. After centrifugation at 15,000 g for 20 min, the pellet was discarded and the supernatant was dialyzed against the phosphate buffer overnight. The dialysate was centrifuged to remove any precipitate and then applied to a Affi-Gel Blue affinity column equilibrated with the two volume of 30 mM potassium phosphate buffer containing 30 mM NaCl, 2 mM $MgCl_2$ and 0.5 mM $CoCl_2$. After washing with the buffer, the enzyme was eluted with a linear gradient from 0 to 0.64 mM NADH in the buffer (total volume 200 m*l*). Fractions showing ADH activity were pooled and dialyzed against 10 mM phosphate buffer containing 0.5 mM $CoCl_2$. The enzyme solution was subsequently loaded to hydroxylapatite column (2.5 × 8 cm). The enzyme was eluted with 2 volumes of the buffer by increasing the concentration of potassium phosphate from 10 to 150 mM at constant pH.

Enzyme purification with cell extract of *Z. mobilis*

Until the step of the Affi-Gel Blue column chromatography, the same procedures were done for purification of the enzyme ZADH-II from *Z. mobilis* without ammonium sulphate fractionation step. Finally, the active fractions from Affi-Gel Blue column were dialyzed against 15 mM potassium phosphate buffer containing 0.5 mM $CoCl_2$ and followed by placing on DEAE-Sephadex A-50 columnn. The enzyme was eluted with a linear gradient from 15 to 150 mM potassium phosphate in the buffer (pH 7.0).

Assay of enzyme activity

Assay of ADH activity was performed by measuring the quantity of NADH produced at 340 nm. The reaction mixture contained 333 mM ethanol, 10 mM NAD^+, enzyme solution and 50 mM Tris-HCl (pH 8.5) with 0.5 mM $CoCl_2$ to give a final volume of 3.0 m*l*. The reaction was initiated by the addition of enzyme solution. One unit of enzyme activity is defined as a nanomole of the product formed per minute of incubation at room temperature.

Protein analysis

Protein concentration was determined by the method of Lowry *et al.* (7) or Comassie blue method (8) with bovine serum albumin (BSA) as a standard. Polyacrylamide gel electrophoresis (PAGE) of the purified enzyme was performed in gel containing 10% acrylamide and 0.1% SDS according to Laemmli (9).

Results and Discussion

Enzyme purification

During the purification it was noticed that the enzyme was very unstable. However, the stability of enzyme was greatly enhanced in the presence of cobaltous chloride. The cobaltous chloride was therefore added to the buffer used in the purification.

The overall purification steps of the ZADH-II from the cell extracts of *E. coli* (pADS93) and *Z. mobilis* were summarized in Table 1. The final enzyme preparation from *E. coli* (pADS93) had a specific activity 44-fold higher than that of crude extract and represented a yield of 71%. The active fractions eluted from Affi-Gel Blue column by NADH (0.15-0.22 mM) showed two major protein bands in the SDS-PAGE. The active fractions were subjected to hydroxylapatite column to purify the protein further. The purified enzyme displayed a single protein component on 10% polyacrylamide gel containing 0.1% SDS (Fig. 1). In addition, ZADH-II was purified from cell-extract of *Z. mobilis*. The cell-free extract of *Z. mobilis* was so viscous that precipitation of proteins was not occurred with ammonium sulphate. Therefore, the cell-free extract was directly charged on the column without pretreatment. Since the two isozymes of *Z. mobilis* showed different affinities for NADH, two isozymes were separated by Affi-Gel Blue column chromatography. The active fractions for ZADH-II were pooled, and followed by DEAE-Sephadex A-50 chromatography to give a single protein band identical to that obtained from *E. coli* (pADS93) on SDS- polyacrylamide gel (Fig. 1).

Table 1. General scheme for the purification of alcohol dehydrogenase from *E. coli* (pADS93) and *Z. mobilis*

Step	Vol (m*l*)	Activity (units/m*l*)	Protein (mg/m*l*)	Specific activity (units/mg protein)	Recovery (%)
E. coli (pADS93)					
Crude extract	20	18.9	11.2	1.7	100
$(NH_4)_2SO_4$ fractionation	30	11.2	4.9	2.3	88.8
Affi-Gel Blue	32	9.4	0.2	47	79.6
Hydroxylapatite	12	22.3	0.3	74.3	70.8
Z. mobilis					
Crude extract	20	24.3	20.2	1.2	100
Affi-Gel Blue	18	20.8	0.52	40	86.1
DEAE-Sephadex A-50	24	12.0	0.19	62	66.2

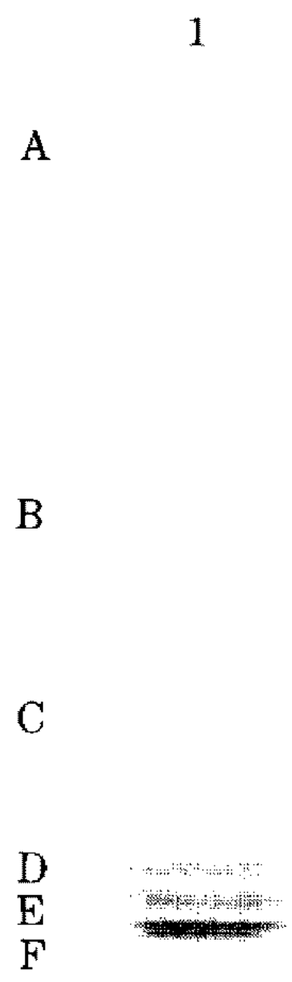

Fig. 1. SDS-polyacrylamide gel electrophoresis estimating molecular weight of subunits of the purified enzyme.

A mixture of standard protein subunits was run in lane 1 to enable the molecular weight of ZADH-II subunit. These standards are as follows: A, Bovine albumin (M_r 66,000); B, Egg albumin (M_r 45,000); C, Pepsin (M_r 34,700); D, Trypsinogen (Mr 24,000); E, β-lactoglobulin (Mr 18,400); F, Lysozyme (Mr 14,300). Lane 2, the purified ZADH-II from *Z. mobilis*; 3, the purified ZADH-II from *E. coli* (pADS93).

Molecular weight determination

SDS-PAGE of the purified enzymes yield a single protein band corresponding to molecular weight 40,000 ± 1,000 (Fig. 1), which agrees with that (M_r 39,999) of enzyme predicted by nucleotide sequence of *adhB* gene (6). Molecular weight of the intact enzyme was identified to be 170,000 by Sephadex G-200 chromatography (Fig. 2), suggesting that the enzyme is a tetramer constructed from identical subunits. However it was different from the results reported by other groups (2, 11), though it is unclear what caused this difference.

Effect of pH

Over a range of pH values, the initial velocity of both the forward (ethanol oxidation) and reverse (acetaldehyde reduction) reactions was tested using the purified enzyme. The results of the effect of pH on enzyme activity are displayed in Fig. 3. The enzyme shows broad activity peak for the reverse reaction with the optimum between pH 7.5 and 8.5, but relatively narrow activity peak for the forward reaction with the optimum at pH 10.0.

Effect of metal ions

The activity of ZADH-II prepared from *E. coli* (pADS93) was affected by divalent metal ions as reported by Scopes (10). In the presence of EDTA, the purified enzyme was almost inactivated within 30 min as shown in Fig. 4. When the divalent metal ion was added to EDTA-treated enzyme the activity of the EDTA-treated enzyme was restored by ferrous or cobaltous ion, but not by zinc ion. Ferrous ion was more effective than cobaltous ion in restoring the activity of enzyme, but the activity was immediately decreased by oxidation of ferrous ion on the ambient condition. Since dithiothreitol protects ferrous ion

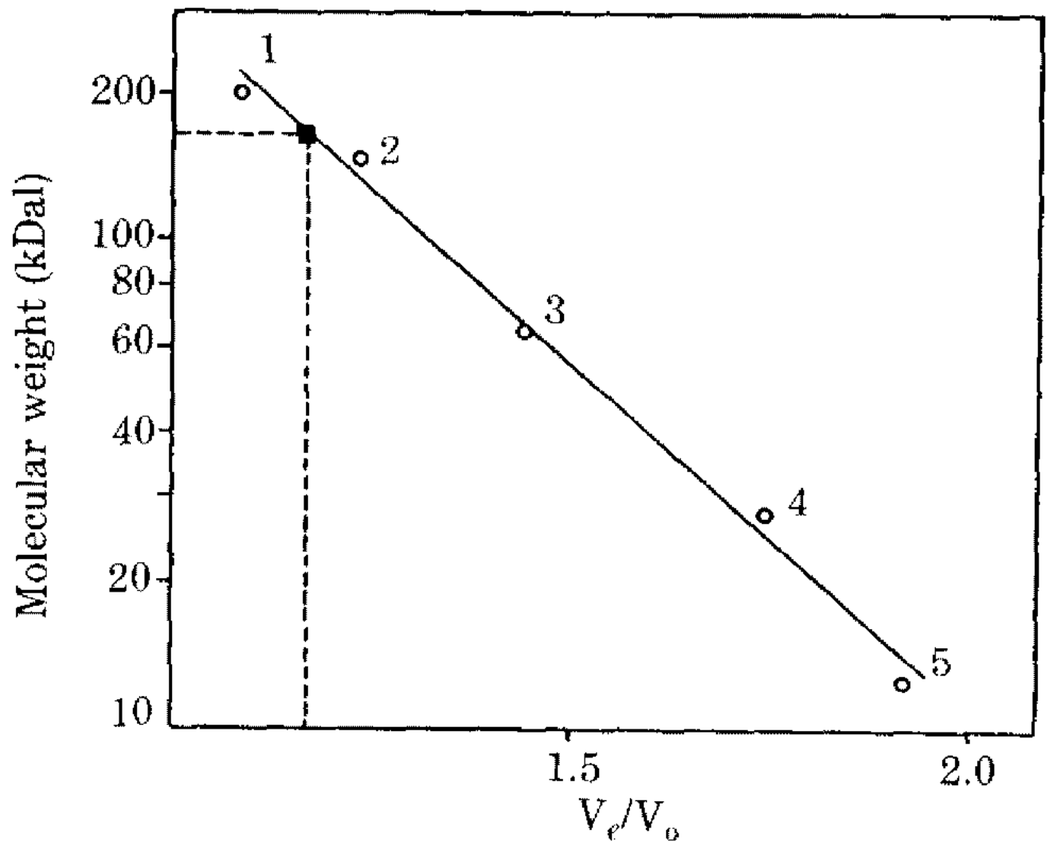

Fig. 2. Molecular weight determination of ZADH-II on a Sephadex G-200 column chromatography (1.6×90 cm). The V_o was determined with blue dextran. The V_e of proteins was determined by absorbance at 280 nm. The standards are as follows: 1, β-amylase of sweet potato (M_r 200,000); 2, Yeast ADH (M_r 150,000); 3, Bovine albumin (M_r 66,000); 4, Carbonic anhydrase of bovine erythrocyte (M_r 29,000); 5, Cytochrome c of horse heart (M_r 12,400).

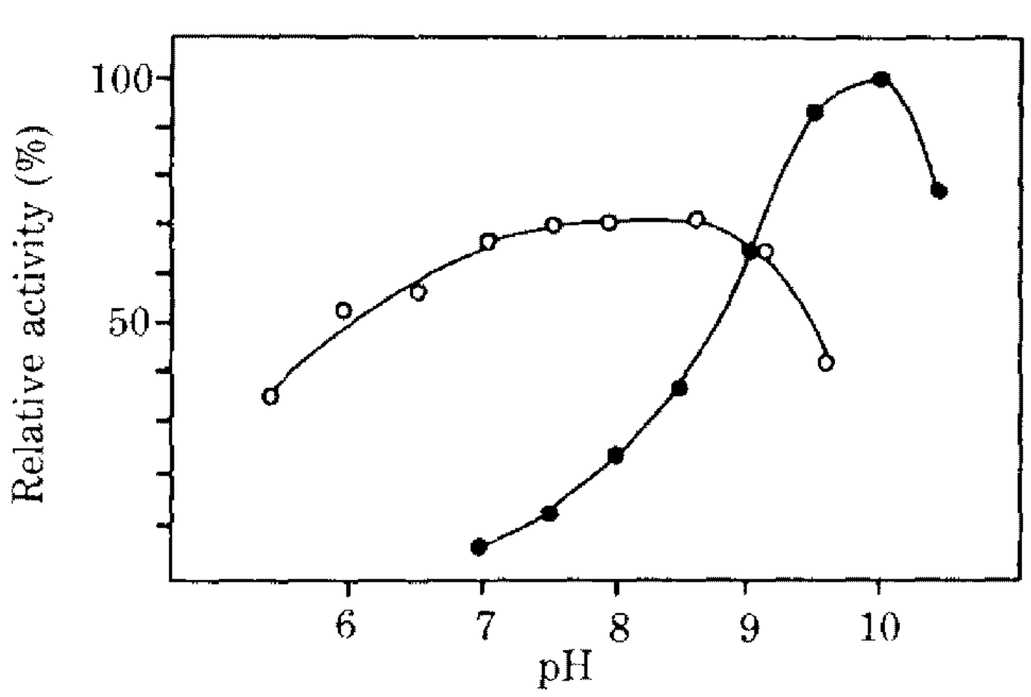

Fig. 3. The effects of pH on the activity of ZADH-II. Buffer systems used for the whole range of pH are: 4.0-5.5, sodium-acetate buffer; 5.5-8.0, Clark-Lubs buffer; 7.5-8.5, Tris-HCl buffer; 8.5-10.5, glycine-NaOH buffer; 10.5-12.0, phosphate buffer. ●, ethanol oxidation; ○, acetaldehyde reduction.

from oxidation the activity was sharply increased up to two times of the original level, and maintained stably in the presence of ferrous ion plus dithiothreitol.

Kinetic parameters

Table 2 shows the kinetic parameters of both the forward and reverse reactions which were evaluated with ethanol and acetaldehyde as the respective substrates. All conditions were as described for the standard assay with changes in the concentration of sub-

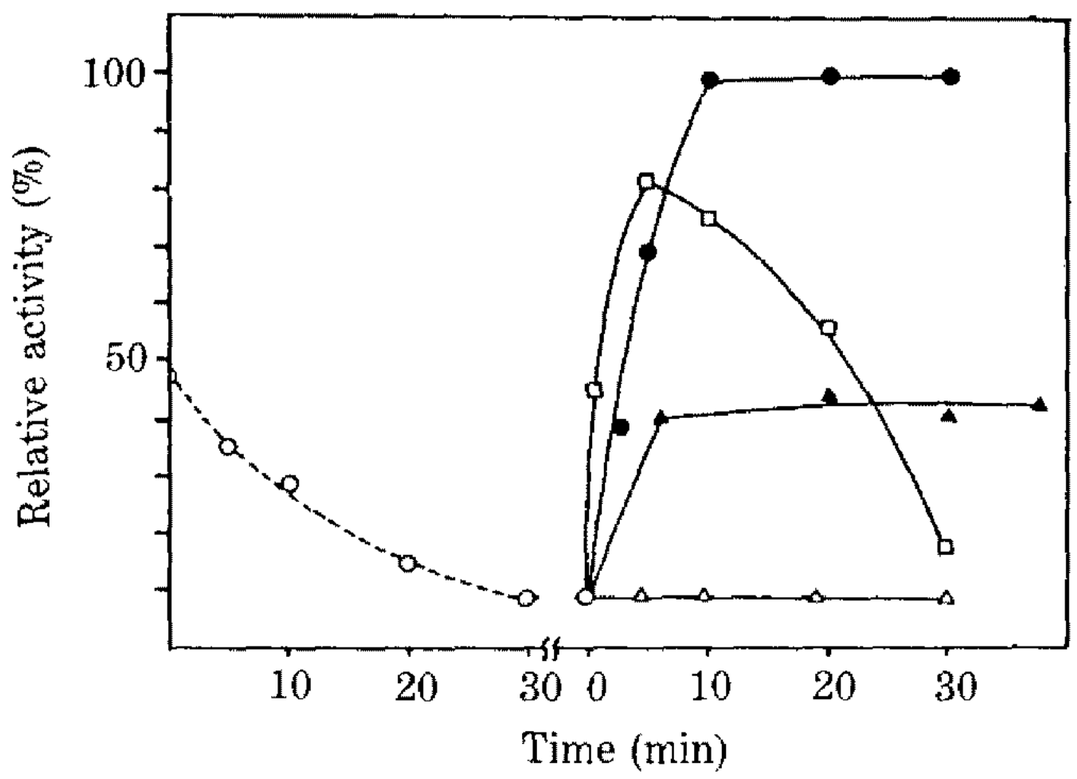

Fig. 4. Effects of metal ions on the activity of ZADH-II. Purified enzyme was treated with 25 mM EDTA (○) at zero time. After 30 min, excess divalent metal ions were added to give the final concentrations of 12.5 mM for ammonium ferrous sulfate and 5 mM for dithiothreitol (●), 12.5 mM for ammonium ferrous sulfate (□), 250 mM for cobaltous chloride (▲), and 250 mM for zinc chloride (△).

Table 2. Kinetic constants for the *Z. mobilis* alcohol dehydrogenase-II

Substrates	Ethanol	NAD^+	Acetaldehyde	NADH
K_m (M)	$1.2\times10^{-}$	5.1×10^{-5}	8.7×10^{-4}	3.5×10^{-5}

strates. The K_m values for the substrates were determined by extrapolation of the $1/V_{max.app}$ taken from the double-reciprocal plots described by Florini and Vestling (12).

요 약

*Zymomonas mobilis*의 알코올 탈수소 효소 유전자가 클로닝된 대장균 형질전환체의 세포 추출물로부터 알코올 탈수소 효소를 분리정제하였다. 형질전환된 *Escherichia coli* (pADS93)가 생산하는 *Z. mobilis* 유전자 유래의 알코올 탈수소 효소는 분자량이 40,000인 동일한 4개의 subunits로 구성된 tetramer임이 밝혀졌으며 이것은 *Z. mobilis* 세포 추출물로부터 분리한 알코올 탈수소 효소와 동일하였다. 이 효소의 정반응(ethanol 산화)은 pH의 영향을 많이 받으며 최적의 pH는 10.0이었고 역반응(acetaldehyde 환원)에서는 최적의 pH가 7.5~8.5이었지만 pH에 따라 크게 영향을 받지는 않았다. 한편 기질인 ethanol과 NAD에 대한 K_m값은 각각 1.2×10^{-1} M과 5.1×10^{-5} M로 나타났으며 acetalde-

hyde에 대한 K_m값은 ethanol에 대한 K_m값보다 매우 낮았다.

References

1. Neale, A.D., R.K. Scopes, J.M. Kelly and R.E.H. Wettenhall: *Eur. J. Biochem.* **154,** 119 (1986).
2. Wills, C., P. Kratofil, D. Londo and T. Martin: *Arch. Biochem. Biophys.* **210,** 775 (1981).
3. Das, K., K. Kadoda, S. Kinoshita and H. Taguchi: *Annu. Rep. Int. Cent. Co-op. Res. Dev. Microbiol. Eng.* **4,** 436 (1981).
4. Conway, T., G.W. Sewell, Y.A. Osman and L.O. Ingram: *J. Bacteriol.* **169,** 2591 (1987).
5. Yoon, K.H., B.S. Shin and M.Y. Pack: *Kor. J. Appl. Microbiol. Bioeng.* **17,** 301 (1989).
6. Yoon, K.H. and M.Y. Pack: *Nucleic Acids Res.* **18,** 187 (1990).
7. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall: *J. Biol. Chem.* **193,** 265 (1951).
8. Sedmak, J.J. and S.E. Grossberg: *Anal. Biochem.* **79,** 544 (1977).
9. Laemmli, U.K.: *Nature* **227,** 680 (1970).
10. Scopes, R.K.: *FEBS Lett.* **156,** 303 (1983).
11. Hoppner, T.C. and H.W. Doelle: *Eur. J. Appl. Microbiol. Biotechnol.* **17,** 152 (1983).
12. Florini, J.R. and C.S. Vestling: *Biochim. Biophys. Acta* **25,** 575 (1957).

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