

Purification and biochemical properties of rice alcohol dehydrogenase

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Abstract. Alcohol dehydrogenase (ADH) isozyme pattern of the imbibited seeds and the water-logging treated shoots were similar. The dominant enzyme activity was the slowest band in electromobility. This enzymes was extracted from 24 hours-imbibited seeds and purified 76 fold with a 17% yield by sequential steps of ammonium sulfate fractionation and chromatography on Sephadex G-150, DEAE Sephadex A-25 and Blue Sepharose 6B. Molecular weight of the purified dominant enzyme was 86,000 daltons and it contained two subunits of same size. The subunit weight was 46,000 daltons. The optimum pH for ethanol oxidation was pH9.5 and for acetaldehyde reduction was pH 6.5. The Km values for ethanol, NAD⁺, acetaldehyde and NADH were 47.3 mM, 51.7 μ M, 7.05 mM and 31.1 μ M respectively. The enzyme was stable in neutral pH and the enzyme activity was fully retained at 40°C for at least 60 min.

Key words: Alcohol dehydrogenase; Isozyme; Purification; *Oryza sativa* L.

Introduction

Alcohol dehydrogenase (ADH, EC 1.1.1.1) is the major enzyme involving in the process of anaerobic fermentation of plants. The enzyme activity is induced when the plant grows under the stress of anoxia (Davies, 1980). Genetic and molecular biological studies on the ADH have been done in barley (Hanson *et al.*, 1984; Harberd and Edwards, 1982, 1983) and maize (Sachs and Freeling, 1978; Hake *et al.*, 1985; Gerlach *et al.*, 1982). Rice is the most anoxia tolerant plant among all cereals. Rice seeds germinate with high metabolic activities

under anoxia and coleoptiles even grow in an environment containing little or no detectable amounts of oxygen (Mocquot *et al.*, 1981; John and Greenway, 1976). More recently, a few studies specially addressing to the physiological function of ADH under anaerobic induction have been reported (Shimomura and Beevers, 1983; Kang *et al.*, 1986).

This enzyme have been purified and characterized from various plant materials such as maize (Felder *et al.*, 1973), peanut (Pattee and Swaisgood, 1968), tea seed (Hatanaka *et al.*, 1974) and cultured rice cells (Igaue and Yagi, 1982). In this work we report that ADH was purified from imbibited seed, biochemical properties of the purified enzyme, and the possible physiological role of this enzyme under

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anaerobic stress.

Materials and Methods

Rice seeds of *Oryza sativa* cv. Tainung 67 was donated by Institute of Botany, Academia Sinica, and the hulled seeds of Tainung 67 were obtained from the local market. Sephadex G-150, DEAE Sephadex A-25, Blue Sepharose CL-6B and molecular weight markers were purchased from Pharmacia, Sweden; EDTA, NAD⁺, NADH, polyvinyl pyrrolidone (PVP), nitro blue tetrazolium and N, N-methylene-bis-acrylamide were purchased from Sigma, St. Louis, USA; acetaldehyde, acrylamide, zinc chloride, sodium dodecyl sulfate, Coomassie Brilliant Blue G-250 were purchased from Merck, Darmstadt, West Germany; dithiothreitol, yeast ADH were purchased from Boehringer Mannheim GmbH, West Germany.

Treatments of Rice Seeds

Seeds were sterilized by 5min exposure in a 5% sodium hypochlorite solution followed by 30 min, washing under running water and then rinsed twice with deionized water (ddw). The washed seeds were germinated in a plastic box (45×30×10 cm³) containing one sheet of filter paper saturated with ddw.

For waterlogging experiments, the seedlings were transferred to a sterilized flask (250 ml) which was filled with 200 ml of ddw and covered with aluminum foil. For large amount of ADH extraction, 1 kg of hulled seeds were sterilized by a 2% sodium hypochlorite solution for 5min. and washed, rinsed as described before. The seeds were then submerged in a 3-liter beaker filled with ddw. All the containers were placed in darkness in a 30% growth chamber.

Enzyme Purification

After 24 hours imbibition, the hulled seeds

were rinsed with ddw and homogenized in a prechilled Waring blender with 1 liter of extraction buffer containing 50mM Tris-HCl buffer (pH 7.6), 0.5mM zinc chloride, 10mM 2-mercaptoethanol and 10g of insoluble PVP. Homogenates were passed through four layers of cheesecloth and centrifuged at 9,300×g for 30 min at 4%. The supernatant was used as crude extract.

Ammonium sulfate fractionation was proceeded and the precipitate in the concentration between 35 to 60% was collected and re-suspended in 40 ml of buffer A (50mM Tris-HCl, 0.5mM zinc chloride, 10mM 2-mercaptoethanol, pH 7.6). The enzyme suspension was dialyzed against buffer A overnight and then was centrifuged. The supernatant was carefully loaded on a sephadex G-150 column (2.5×75 cm) and eluted with buffer A at the flow rate of 0.5 ml/min.. The enzyme fractions were pooled and loaded on a DEAE sephadex A-25 column (2.5×30 cm). The column was first washed with buffer A until the eluate showed no more absorbance at 280 nm and then eluted with buffer A containing linear gradient of 0 to 0.3M potassium chloride. Fractions with enzyme activity were pooled and pumped into a blue sepharose 6B column (2×10 cm). The column was first washed completely with buffer A and then eluted with buffer A containing 2mM NAD⁺. The enzyme fractions were pooled and concentrated with Amicon Centricon-10. The concentrated enzyme solution was stored in liquid nitrogen.

Assay of Enzyme Activity

To determine the activity of ADH for ethanol oxidation (Bonnichsen and Brink, 1955), the assay mixture contained 0.1 M glycine-NaOH buffer pH 9.0, 75 mM ethanol and 0.26mM NAD⁺. After the addition of enzyme solution to the mixture, the initial rate of NAD⁺ reduction

was measured at 340 nm with a Gilford 250 spectrophotometer. One unit of ADH activity was defined as the amount which catalyzed 1 μ mole of NAD⁺ per min. For acetaldehyde reduction the assay mixture contained 0.1M phosphate buffer pH6.5, 20mM acetaldehyde and 0.05mM NADH. The initial rate of the NADH oxidation was measured at 340nm. Catalase activity was determined according to the method of Chance and Maehly (1955). The reaction mixture contained 63mM potassium phosphate buffer pH7.0 and 0.075% hydrogen peroxide. The initial rate was determined at 240 nm. Protein concentration was measured by the method of Lowry *et al.* (1951), with crystalline bovin serum albumin as the standard.

Gel Electrophoresis

Native slab gel electrophoresis was conducted as described by Ames (1974). A constant current of 25mA was applied for 4 hours at 4°C. Gels were stained either for ADH activity by the method of Scandalios (1967) or for protein with Coomassie Brilliant Blue R-250. The molecular weight of the subunit was estimated by the method of Weber and Osborne (1969) with SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Results

Electrophoretic Analysis of Rice ADH

The growth of etiolated seedling involved mainly the cell elongation, they were easily stimulated by environmental factors such as anaerobic stress. For this reason we have compared the isozyme pattern from tissues under normal growth and reduced oxygen content. Shoots of etiolated seedlings grown in air had low ADH activity. The activity was

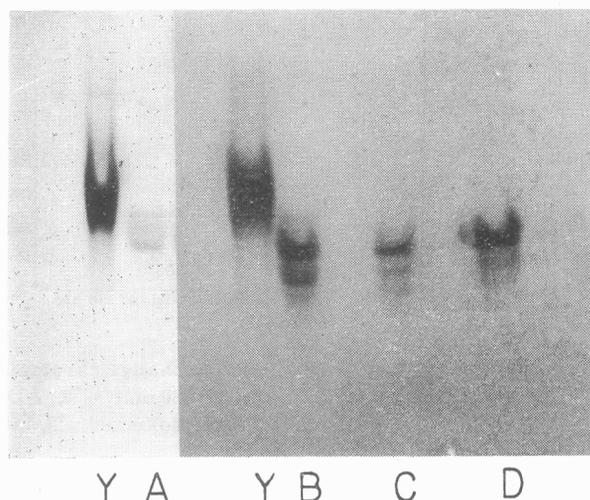


Fig. 1. ADH isozymes of rice tissues. Isozymes were separated by native PAGE and stained for enzyme activity. A. extract from shoots of 4 day air grown seedlings and the extract was concentrated fifteen fold. B. extract from 24 hours imbibed seeds. C. extract from shoots of 4 day air grown and 12 hours waterlogging seedlings. D. extract from shoots of 4 day air grown and 72 hours waterlogging seedlings. Y. yeast ADH.

too low to be detected by staining the gel after electrophoresis. Even the sample was concentrated to fifteen folds, there was only a faint single band (Fig. 1 A). In the sample of imbibed seed, there was a faint fast moving band in front of two main bands (Fig. 1 B). ADH activity of seedlings was induced by waterlogging. Three isozymes were detected after 12 hours of treatment (Fig. 1 C). Prolonged treatment increased the ADH activity of the isozyme species with the slowest mobility (Fig. 1 D). It appears that the slow moving isozyme species represent the main activity induced. A similar isozyme pattern was also observed in the imbibed seeds. The imbibed seeds seems to be a good source for large quantitative isolation of ADH.

Enzyme Purification

About 90% of the enzyme activity in crude

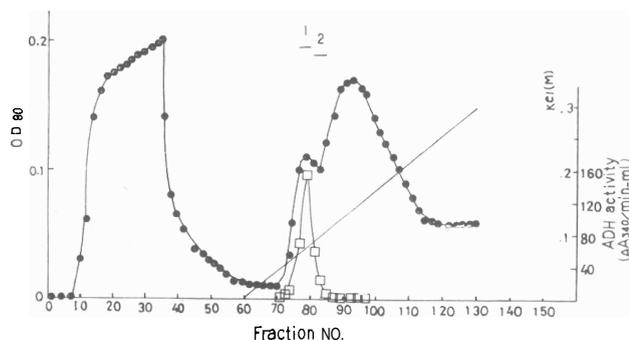


Fig. 2. DEAE-Sephadex A-25 anion exchange chromatography. ●, absorbance at 280 nm; □, ADH activity. 1, peak fractions collected; 2, late shoulder fractions collected.

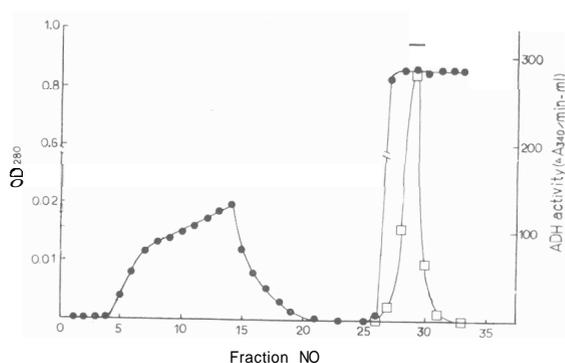


Fig. 3. Blue Sepharose CL-6B affinity chromatography. ●, absorbance at 280 nm; □, ADH activity.

extract was recovered from 35 to 60% ammonium sulfate fractionation. Chromatography of the enzyme on column of DEAE sephadex A-25 is shown in Fig. 2. According to the absorbance at 280 nm, a large amount of proteins was eliminated by the chromato-

graphy, but a significant amount of enzyme activity was also lost inevitably in the process, even the pH and ionic strength of the buffer had been changed. Figure 3 shows the affinity chromatography of the enzyme on Blue Sepharose 4B column. This step was more effective in enzyme purification. After the affinity chromatography seventy six-fold purification of the enzyme was achieved. A final yield of enzyme activity was 17% of the crude enzyme extract, and the specific activity of the purified enzyme of 87.7 units per mg protein was obtained (Table 1). In order to understand the separation of isozymes during purification, ADH isozymes was checked on the electrophoretic gel by activity stain. The late shoulder fractions collected from anion exchange chromatography exhibited very low ADH activity (see Fig. 2) which was further concentrated for isozyme analysis. In Fig. 4 the crude extract and the late shoulder fractions showed in lane A and C were concentrated twenty and thirty fold, respectively. In this run, the crude extract did not show off the fast moving band, the reason was supposed to be less sample mass was loaded. A minor isozyme band was always associated with the main band during purification. The isozymes from the concentrated preparation of late shoulder fractions (Fig. 4C) showed a relative higher proportion of minor band in comparison with the isozymes from peak fraction (Fig. 4B) on the basis of total stain intensity. Therefore, low enzyme activity of late shoulder

Table 1. Summary of the ADH purification

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification (fold)	Recovery (%)
Crude	840	1,710.03	1,495.20	1.14	—	100
Ammonium Sulfate	40	1,534.64	609.66	2.52	2.20	89.74
Sephadex G-150	150	1,496.84	443.40	3.38	2.95	87.53
DEAE-Sephadex	60	551.22	61.44	8.97	7.84	32.23
Affinity chromatography	30	288.66	3.29	87.74	76.71	16.88

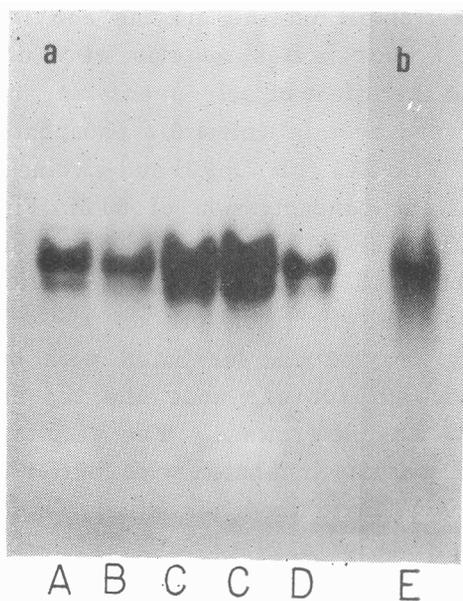


Fig. 4. ADH isozymes and purified enzyme protein separated by native PAGE. After electrophoresis the gel was cut into two parts (a, b): a gel was stained for ADH activity; b gel was stained with Coomassie Brilliant Blue. Lane A, concentrated crude extract; Lane B and C were the fractions recovered from ion exchange chromatography of Fig. 2, in which lane B was the peak region of the collection 1, lane C was the late shoulder region of the collection 2 (30 folds concentrated); Lane D, enzyme purified by the affinity chromatography; Lane E, same sample as D, but stained with Coomassie Brilliant Blue.

fractions might be due to the higher proportion of minor isozyme. A possible explanation is that this minor isozyme could be derived from the major enzyme undergone some unknown modifications resulting in lower activity and higher negative charge. Because the buffer used for purification contained 10mM 2-mercaptoethanol, modification of the enzyme molecules with respect to oxidation of -SH groups seems unlikely.

After affinity column chromatography the minor band of isozyme was not completely eliminated. Thus, the gel lane which was cut from the same gel and stained by Coomassie Brilliant Blue shows a single broad band (Fig.

4E). The molecular weight of the enzyme was determined by Sephadex G-150 gel filtration (Fig. 5). The estimated molecular weight of rice ADH is 86,000, which was close to the molecular weights of other plant ADHs reported (Leblova *et al.*, 1977). Molecular weight of the subunits was estimated by SDS-polyacrylamide gel electrophoresis (Fig. 6). A single band with a molecular weight of 46,000 was shown on the gel. Thus, rice ADH should be composed of two subunits of the same size, which is very similar to the enzyme purified from the cultured rice cells (Igaue and Yagi, 1982).

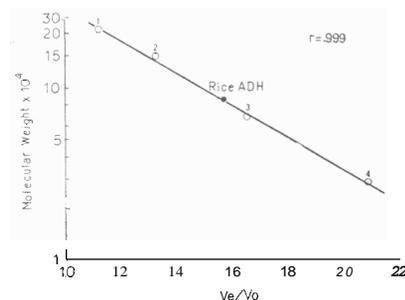


Fig. 5. Estimation of the molecular weight of ADH by Sephadex G-150 gel filtration. 1, catalase; 2, yeast ADH; 3, bovine serum albumin; 4, cytochrome C.

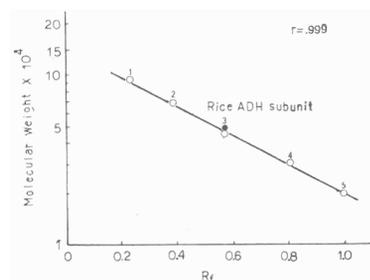


Fig. 6. Estimation of subunit weight of ADH by SDS-PAGE. 1, phosphorylase b; 2, bovine serum albumin; 3, ovalbumin; 4, carbonic anhydrase; 5, soybean trypsin inhibitor.

Biochemical Properties

Affinities for the substrate and coenzyme: Table 2 shows the K_m values of the enzyme for substrate and coenzyme, which were

Table 2. *K_m of alcohol dehydrogenase of rice*

Substrate	<i>K_m</i> (M)
NAD ⁺	5.1735×10^{-5}
Ethanol	4.7364×10^{-2}
NADH	3.1156×10^{-5}
Acetaldehyde	7.0505×10^{-3}

determined by Lineweaver-Burk method. The enzyme activities in the backward reaction with various concentrations of ethanol and NAD⁺ were measured at pH 9.5, while the forward reactions were measured at pH 6.5. The affinity of enzyme for NAD⁺ and NADH is in the same range deduced from the *K_m* values, but the affinity for ethanol is lower than that for acetaldehyde. This suggests that ADH favors to catalyze the forward reaction from acetaldehyde to ethanol, when the equal amount of acetaldehyde and ethanol are present.

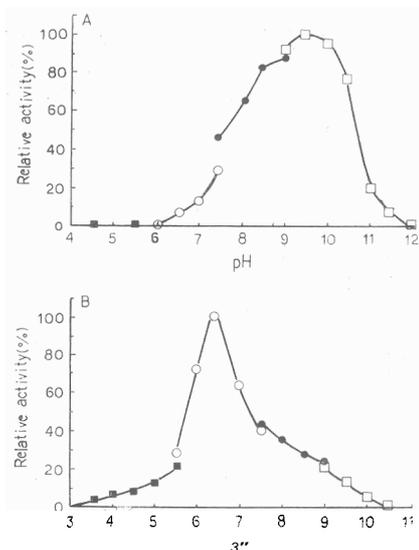


Fig. 7. Effect of pH on the enzyme activity. ADH activities were measured in buffer systems of various pH at 25°C. A, ethanol oxidation; B, acetaldehyde reduction. ■, 0.1 M sodium acetate buffer; ○, 0.1 M sodium phosphate buffer; ●, 0.1 M Tris-HCl buffer; □, 0.1 M glycine-NaOH buffer.

Effect of pH on the enzyme activity and stability: Four buffer systems were used to examine the effect of pH on enzyme activity. They were acetate (pH 4-6); phosphate (pH 5.5-7.5); Tris-HCl (pH 7.5-9.0) and glycine-NaOH (pH 9-12) at concentration of 0.1M. Figure 7 shows that the pH optimum is 9.5 for ethanol oxidation and 6.5 for acetaldehyde reduction. To examine the effect of pH on the enzyme stability, enzyme was incubated with buffers of various pH for one hour and the enzyme activity was determined. The results show that the enzyme incubated with buffer of pH from 6.0 to 9.5 retained full activity (Fig. 8)

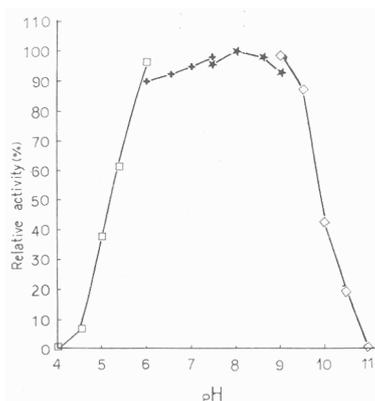


Fig. 8. Effect of pH on enzyme stability. Enzyme was preincubated in buffer systems of various pH at 25°C for 60 min. After incubation ADH activity was measured in glycine-NaOH buffer, pH 9.0 at 25°C. □, 0.1 M sodium acetate buffer; ○, 0.1 M sodium phosphate buffer; ●, 0.1 M Tris-HCl buffer; △, 0.1 M glycine-NaOH buffer.

Thermostability and substrate specificity: After incubation at 40°C, 50°C and 60°C for various time intervals, the enzyme activities were determined. The enzyme remained fully active at 40°C after 60 min., whereas slight decrease in activity was observed at 50°C. No more enzyme activity could be detected at 60°C after 20 min. (Fig. 9). This thermoeffect on enzyme stability showed the first order reaction.

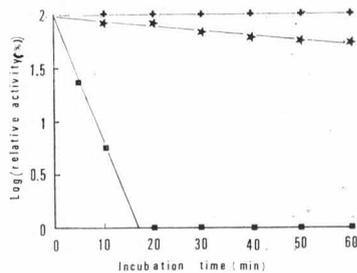


Fig. 9. Effect of temperature on the ADH activity. Enzyme in solution was incubated at various temperature as indicated. An aliquot of enzyme was taken at 10 min interval for enzyme activity assay carried out at 25°C. --○--, 40°C; --★--, 50°C; --■--, 60°C.

The substrate specificity of the enzyme was examined with various alcohols. Except methanol, the relationship between enzyme activity and number of carbon atoms in the primary saturated alcohols manifested in a first order manner, while increasing in the carbon number of the substrate decreased the enzyme activity expressed as relative reaction rate (Fig. 10).

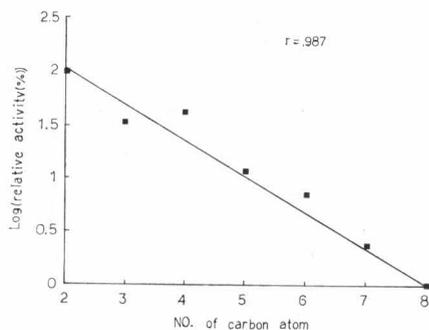


Fig. 10. Relationship of the enzyme-substrate specificity and the number of carbon atoms in the primary saturated alcohols. Primary saturated alcohols of two to eight carbon atoms were used as substrates for ADH activity assay at 25°C.

Discussion

Rice is a flood tolerant plant in cereals and ADH is the one of important enzyme which are induced under anaerobic condition. ADH

activity in the seeds increased with the time up to 24 hours after imbibition, and three isozymes were observed on the zymogram of native gel. Rice cells in culture had a single activity band presented on gel (Igaue and Yagi, 1982). Since cells are undifferentiated in the suspension culture, the isozyme difference among germinated embryo, shoot of seedling and cultured cell could result from the diversity of physiological conditions.

The enzyme purified from imbibited seeds is a dimeric protein with a molecular weight of 86,000 daltons and consists of two subunits of same size, with molecular weight of 46,000 daltons. The molecular weight of ADH of other plants range from 50,000 to 100,000 daltons.

ADH of tea, wheat, maize and cultured rice cells also appear to have dimers (Scandalios, 1967; Pattee and Swaisgood, 1968; Hart *et al.*, 1969; Freeling and Schwartz, 1973; Hatanaka *et al.*, 1976; Igaue and Yagi, 1982).

Purified enzyme is stable in the neutral pH ranged from 6.0 to 9.5. It shows that the enzyme can tolerate slight pH changes in its environment. The enzyme activity was fully retained after incubation at 40°C for 60 min, and the activity decayed at 60°C with the first order rate of $t(1/2)$ of 8 min.. Similar result has also been found by Igaue and Yagi (1982).

Davies (1980) discussed in detail about the metabolic change in plant under anaerobic stress. Robert *et al.* (1984, 1985) has demonstrated that the plant tissue under condition of oxygen deprivation will cause cytoplasmic acidosis consequently. Rice ADH has been shown to be a soluble enzyme localized in the cytosol (Shimomura and Beeves, 1983), change of pH in the cytoplasm should affect directly on the catalytic reaction of ADH according to the pH optimum of enzyme. Rice ADH shows pH optimum at 9.5 for ethanol oxidation and at 6.5 for acetaldehyde reduction (Fig. 7). Cytoplasmic acidosis of plant tissue would be

favorable to the acetaldehyde reduction catalyzed by, ADH. Therefore, alcohol fermentation could be accelerated in tissue in order to meet the energy requirement under anaerobic stress.

Under anaerobic treatment a small amount of ethanol was detected in rice tissues, while most of the ethanol produced was found in the growth medium (Bertani *et al.*, 1980). Concomitant increase of ADH activity and the ethanol production was observed in culture of rice cells, and most of the ethanol produced was diffused into the culture medium (Igaue and Yagi, 1982). It seems that ethanol released from the tissues should be a possible way to avoid ethanol toxicity during anaerobiosis. It is interesting to understand the induction mechanism in rice and the behavior of enzyme to the physiological adaptation under anaerobic stress.

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水稻乙醇去氫酶的純化及其生化性質

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浸水24小時的去殼稻米和淹水幼苗的幼莖，其乙醇去氫酶的異構酶帶非常相似，其中以泳動最慢的酶帶活性最強。從浸水24小時的去殼稻米中萃取乙醇去氫酶。酶萃取液經過硫酸銨的分割；Sephadex G-150, DEAE Sephadex A-25 及 Blue Sepharose 6B 的管柱層析等步驟的處理，得到76倍純化的酶溶液，其回收率為百分之十七。酶的分子量為 86,000 達爾頓，含有兩個相等次體，其分子量為46,000達爾頓。酶對乙醇氧化反應的最適當 pH 值為 9.5，但對乙醛的還原反應則在 pH 6.5 最佳。

對於不同受質、輔酶所測得的 K_m 值分別是乙醇，47.3 mM； NAD^+ ，51.7 μM ；乙醛，7.05 mM； $NADH$ ，31.1 μM 。酶分子在中性 pH 下非常安定，而且在 40°C 溫度下至少在一小時內酶活性完全不受影響。