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Leaf Alcohol XX[1]: Alcohol:NAD Oxidoreductase from Tea Seeds

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The enzyme alcohol:NAD Oxidoreductase from tea seeds was isolated and purified about 5,500-fold from crude extract and specific activity of the purified enzyme was about 34,000 unit/mg against allyl alcohol. In addition it was confirmed that the enzyme consisted of two isozymes. The enzymes catalyzed the reduction of leaf aldehyde to the corresponding alcohol.

INTRODUCTION

Leaf alcohol and leaf aldehyde are widely distributed in green leaves of shrubs, in fruits, berries and vegetables, accompanied by other related isomeric alcohols as shown in Table[2,3] and are responsible for the characteristic green leaf odor, hence the names. They both are of biogenetic interest because they can be looked upon as constituting fragmental structure of jasmone, violet aldehyde, cucumber alcohol and other aroma of plant origin.[3]

Recently they were found in some insect excretions as functioning attractant or repellent as shown in Table.[3]

The earlier works of leaf alcohol and leaf aldehyde have succeeded and developed by S. Takei, M. Ohno and A. Hatanaka *et al.* since 1931.[3]

On the investigation of the enzymatic oxidation mechanism of leaf alcohol, we have reported about alcohol dehydrogenase from *Escherichia coli K*-12 and *Leuconostoc mesenteroides*.[4~6]

In a process of the biosynthesis of leaf alcohol in higher plants, it may be proposed that linolenic acid was a precursor of leaf alcohol as shown in scheme 1 supported by the following experimental results: a) From enzymatic part; (1) Lipoxygenase was isolated and purified from tea seeds that catalyzed the oxygen-addition to C-13 of linolenic acid to the peroxidic compound.[7] (2) Alcohol dehydrogenase was isolated from each of fresh tea leaves and tea seeds as described in this paper. b) From non-enzymatic part; (1) Isomers of leaf alcohol, *E*-hex-3-en-1-ol, *Z*-hex-2-en-1-ol and *E*-hex-2-en-1-ol, were isolated from fresh tea leaves.[2] (2) Photochemical and thermal isomerization of leaf aldehyde to *Z*-hex-2-en-1-al and *Z*-hex-3-en-1-al was investigated.[2]

This paper deals with the purification and properties of alcohol dehydrogenase from tea seeds that holds the important clue on the biosynthesis of leaf alcohol. On the alcohol dehydrogenase in higher plants, for the understanding of the biosynthesis of the volatile compounds such as alcohol, aldehyde and ketone, the enzyme from peas has been investigated by C. E. Eriksson *et al.*[8] but its specific activity was about 186.6 unit/mg very low for the crude enzyme.

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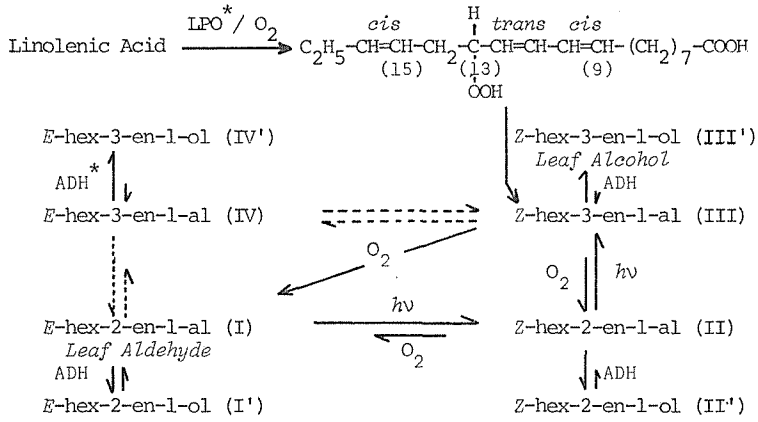
Scheme 1.



* ADH: Alcohol:NAD Oxidoreductase from each of fresh Tea Leaves and Tea Seeds.
LPO: Lipoxygenase from Tea Seeds.

EXPERIMENTAL

The enzyme was extracted from each of fresh tea leaves and tea seeds as enzyme source. But in this paper the latter was used, because the both crude enzymes showed the similar properties and during the process of purification of the ADH* from fresh tea leaves, the enzyme activity was extremely lost by the disturbance of polyphenol.

Extraction of enzyme

The purification procedure was performed according to the following scheme 2. The seeds (500=330 g) were soaked overnight in water. The seeds were ground into powder with a chilled acetone in Homo-mixer. The powders were defatted by washing it with an excess of cold acetone on Buchner funnel. After being dried in a desiccator under vacuum, the enzyme was extracted from acetone-dried powder with 0.05 M Tris-HCl buffer (pH 7.2) containing 10 mM 2-mercaptoethanol at 4°C. The slurry was filtered through cheesecloth and then centrifuged at 12,000 rpm for 10 min at 0°C.

Ammonium sulfate fractionation

Step I. The supernatant solution was precipitated by addition of solid ammonium sulfate to 60% saturation at 4°C and then centrifuged down 20,000×g for 15 min at 0°C. The precipitate obtained was dissolved and then dialyzed against the same buffer.
Step II. The supernatant solution was futher fractionated by ammonium sulfate precipitation. Inactive material was precipitated at 30% saturation and then the enzyme was precipitated by increasing the salt concentration to 60% saturation. The precipitate was dissolved and dialyzed.

* Abbreviation: ADH for Alcohol: NAD Oxidoreductase

Scheme 2. Purification Procedure of Alcohol Dehydrogenase

Tea Seeds
| soaked in water overnight
Acetone powder
|
Extraction
| extracted with the buffer solution
1st Ammonum sulfate fractionation
| the precipitate collected (0-60%)
2nd "
| " (30-60%)
Protamine sulfate treatment
| the precipitate removed
DEAE-Sephadex
| the active fraction collected
G-200 Gel filtration
| the active fraction collected
G-200 "
| "
G-150 "
| "
Hydroxylapatite column chromatography

Protamine sulfate treatment

Protamine sulfate of one third of proteins was added to the supernatant solution and then the precipitate was removed by centrifugation. The supernatant solution was precipitated by addition of solid ammonium sulfate to 60% saturation.

Ion-exchange chromatography

The precipitate was dissolved and dialyzed against 0.05 M Tris-HCl buffer (pH 7.2) containing 10 mM 2-mercaptoethanol and 0.1 M KCl. The enzyme solution was applied DEAE-Sephadex A-50 column (3 cm × 50 cm) which had been equilibrated with the same buffer. The one step elution was performed by applying the same buffer and the active fractions were pooled, concentrated and dialyzed as previously described.

Gel chromatography

Step I. The enzyme solution was passed through Sephadex G-200 gel column (1 × 180 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 7.2) containing 10 mM 2-mercaptoethanol. The elution rate was kept constant at 7.5 ml/hr. The active fractions were pooled and concentrated. The concentrated enzyme solution was passed again

through the same Sephadex G-200 column. The elution rate of rechromatography was performed with 3.5 ml/hr. The active fractions were pooled and concentrated.

Step II. The concentrated enzyme solution was applied to a column (1×180 cm) of Sephadex G-150 which was eluted at 3.5 ml/hr of the elution rate. The fractions containing ADH activities were pooled and concentrated.

Hydroxylapatite column chromatography

The enzyme solution was applied on hydroxylapatite column (1.8×7 cm) equilibrated 0.05 M Tris-HCl buffer (pH 7.2) containing 10 mM 2-mercaptoethanol. The column was flushed with the same buffer and then the stepwise elution was performed in turn 0.1 M Tris-HCl buffer (pH 7.2) containing 10 mM 2-mercaptoethanol, next additional containing 3% ammonium sulfate and then at last containing 10% ammonium sulfate. ADH activity was eluted with last step buffer as shown in Fig. 1.

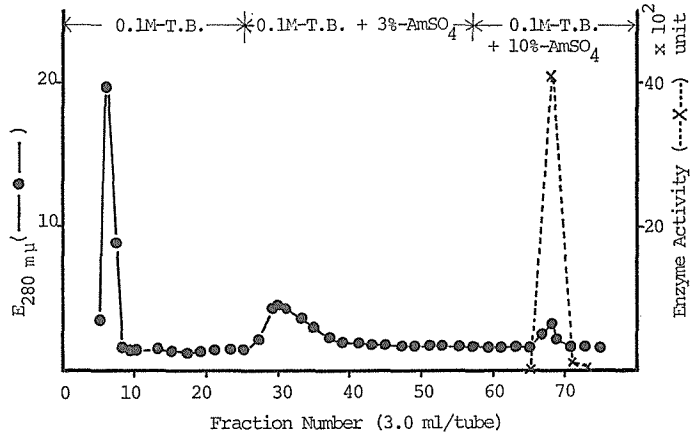


Fig. 1. Stepwise elution pattern of alcohol dehydrogenase on a hydroxylapatite column chromatography

Table 1. Summary of Purification of Alcohol Dehydrogenase

Procedure	Total Protein	Total activity	Total activity	Purification
Crude extract		200,000 unit		
1st Ammoniumsulfate fractionation	10,514 mg	66,000	6.22	1.00
2nd :	7,268	66,700	9.18	1.48
Protamin sulfate	2,844	142,200	50.0	8.04
DEAE-Sephadex	779	154,600	198.3	31.88
Sephadex G-200 Gel filtration	58	77,000	1346.0	216.40
Sephadex G-200 Gel filtration	32	55,100	1714.3	275.45
Sephadex G-150 Gel filtration	15	50,00	3375.3	542.65
Hydroxyl apatite	0.64	20,000	34328.0	5518.34

After the last step, ADH from tea seeds was purified about 5,500-fold from the crude extract and specific activity was about 34,000 unit/mg* against allyl alcohol under the standard assay conditions. The result on each step was summarized in Table 1.

Disc gel electrophoresis

Disc electrophoresis in 7.5% polyacrylamide gel was performed by the modified method of B. J. Davis[9] at pH 9.5. Enzyme activity was located by incubating the gels in a medium containing Nitro Blue Tetrazolium, phenazine methosulfate, allyl alcohol and NAD. In this case, two bands were clearly observed and identified with each of the proteins located by staining with Amido Black 10 B as (a) and (b) as shown in Fig. 2. By the above procedure, it was confirmed that the ADH from tea seeds consisted of two isozymes.

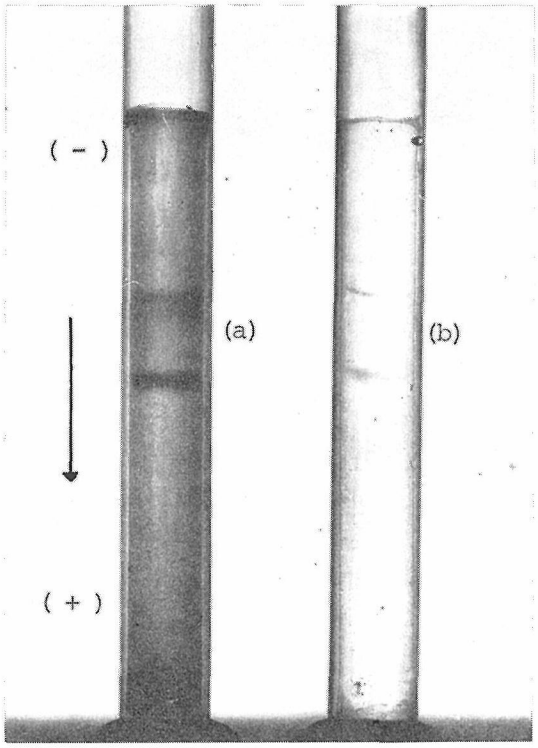


Fig. 2. Polyacrylamide gel electrophoresis of alcohol dehydrogenase

Electrophoresis was carried out in 7.5% polyacryl amide gel for one hr. at 4°C and 2 mA per tube, (a): Enzyme activity bands, (b): Protein bands.

PROPERTIES

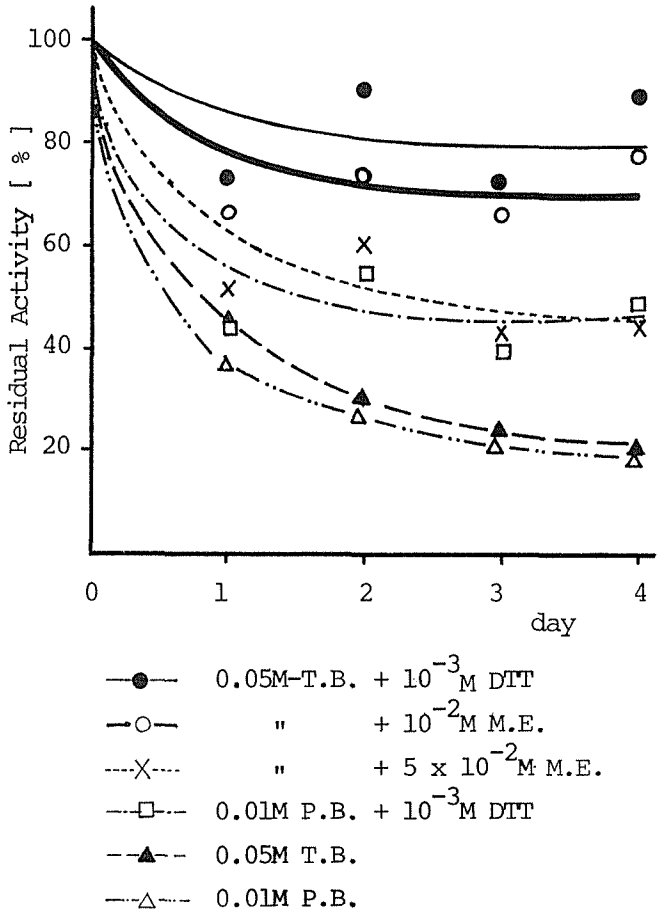
Molecular weight When a mixture of ADH isozymes was subjected to gel filtration

* One unit of the enzyme activity was defined as the amount of 0.001 absorbancy per minute at 25°C.

on Sephadex G-200, the elution peak for these enzymes gave the only one peak. From the gel filtration the molecular weight was estimated as about 150,000.

Co-enzyme. The isozymes ADH from tea seeds required NAD and NADH specifically for the oxidoreduction but not NADP.

Effect of the SH-group on ADH. These were very unstable not only in the absence of SH-compound, but also in the presence of 1 mM 2-mercaptoethanol. But in the presence of 1 mM dithiothreitol or 10 mM 2-mercaptoethanol, these were relatively stable. (Fig. 3)



*T.B.: Tris HCl buffer, P.B.: Phosphate buffer
DTT: Dithiothreitol, M.E.: Mercaptoethanol

Fig. 3. Effect of the SH-group on alcohol dehydrogenase activity

Substrate specificity These enzymes catalyzed very well the reduction of leaf aldehyde to the corresponding alcohol.

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