NAD-dependent Alcohol Dehydrogenase and NADP-dependent Alcohol Dehydrogenase from Strawberry Seeds[†]

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Strawberry seeds are shown to contain at least two alcohol dehydrogenases; one is NAD specific and reacts with ethanol and allyl alcohol, and the other is NADP specific and reacts with benzyl alcohol and geraniol. These two alcohol dehydrogenases were distinguished on disc electrophoresis. Their properties were different each other in ammonium sulfate fractionation, optimum reaction pH and thermostability.

Two hundred and fourteen volatile compounds including twenty carbonyl compounds, forty alcohols and ninety-one esters in strawberries have been isolated and identified.¹⁾ Some aldehydes are known to be converted to their corresponding alcohols followed by further conversion to esters when they are reacted with a whole strawberry fruit.^{2,3)} This suggests that there exists alcohol dehydrogenase (ADH) which catalyzes the interconversion between alcohols and aldehydes or ketones in strawberries.

Alcoholdehydrogenases (ADHs) from pea,⁴⁾ peanut⁵⁾ and valencia orange⁶⁾ react with aliphatic alcohols using NAD as cofactor. The reciprocal interconversion of alcohols and aldehydes has been emphasized by Eriksson⁷⁾ in studies on ADH from peas, in which he reported that aromatic alcohol was also oxidized with NAD as cofactor. Davies^{8,9)} reported that potato tubers contain NAD specific aliphatic ADH and NADP specific aromatic ADH. Hatanaka^{10,11)} and Sekiya¹²⁾ have extensively studied on purification, properties and substrate specificity of ADH from *Thea sinensis* seeds.

The present paper describes the existence and some properties of NAD-dependent ADH and NADP-dependent ADH from strawberry seeds.

MATERIALS AND METHODS

Strawberry seeds. Strawberries (*Fragaria ananassa* Duchesne var. *Donner*) were obtained from a local market. Strawberries were roughly homogenized in water to separate seeds from fruit flesh. The seeds sedimented were collected (approximately 10 g of seeds from 1.5 kg of fresh fruit) and allowed to stand overnight on a filter paper at room temperature to remove excess of water.

Assay of ADH activity. ADH activity was measured spectrophotometrically at 25°C with Beckman DB-GT spectrophotometer. One unit of ADH activity is defined as the amount of enzyme catalyzing the formation of 1 μ mole of NADH or NADPH/min.

Reaction mixture contained (1) 2.5 ml of glycine-NaOH buffer (0.1 M, pH 9.0 or 9.5), 0.1 ml of NAD or NADP (6 mM) and 0.3 ml of ethanol or allyl alcohol or benzyl alcohol (0.3 M) in water, (2) 2.8 ml of geraniol (2 mM) in glycine-NaOH buffer (0.1 M, pH 9.5) and 0.1 ml of NADP (6 mM).

Assay was started by addition of 0.1 ml of enzyme solution to the reaction mixture in a 1 cm cuvette and the initial rate of the reduction of NAD or NADP was measured at 340 nm.

Neither NAD nor NADP was reduced in absence of enzyme or substrate, or by heat-treated enzyme.

Protein concentration. Protein concentration was determined by the method of Lowry *et al.*¹³⁾

Identification of the products. The products were identified by gas chromatography which was programed as follows. Two columns were used; (1) 25% PEG 1000 on Chromosorb W AW (60~80 mesh), 4 m $\times$ 3 mm *i.d.* glass column with column temperature 70°C, (2) 5% Reoplex 400 on Diasolid L (60~80 mesh), 2 m $\times$ 3 mm *i.d.* glass column with column tempera-

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ture 100°C. Column (1) was used for the identification of acetaldehyde and ethanol. Column (2) was used for the identification of benzaldehyde, benzyl alcohol, geranial and geraniol. A 10 μ l of reaction mixture was injected into a Shimadzu GC-4 BM gas chromatograph with FID. Identification was carried out by comparing the retention times of the products with those of authentic samples.

Disc electrophoresis. Disc electrophoresis was performed using 7.5% polyacrylamide gel at pH 8.0¹⁴⁾ with omission of sample gel. A sample of 0.2 ml in 40% sucrose was layered gently on top of the gel. A constant current of 2 mA/tube was applied for 2 hr at 4°C. Enzyme activity was located by incubating the gels in a medium containing 1.0 ml of Nitro Blue Tetrazolium (1.6 mg/ml), 2.0 ml of phenazine methosulfate (0.4 mg/ml), substrate and 0.1 ml of NAD (4.0 mg/ml) or 0.1 ml of NADP (4.8 mg/ml) for 30 min at 25°C in the dark. Substrates added to the medium were (1) 3 ml of 1 M ethanol in glycine-NaOH buffer (0.1 M, pH 9.0), (2) 3 ml of 1 M allyl alcohol in glycine-NaOH buffer (0.1 M, pH 9.0), (3) 3 ml of 0.3 M benzyl alcohol in glycine-NaOH buffer (0.1 M, pH 9.5), and (4) 3 ml of 2 mM geraniol in glycine-NaOH buffer (0.1 M, pH 9.5).

Reagent. NAD and NADP, purity not less than 95%, were obtained from Seikagaku Kogyo Co., Tokyo. Ethanol (99.5%) was obtained from Kanto Chemical Co., Tokyo. Allyl alcohol (99%), geraniol (96%) and geranial and neral mixture (no impurity was detected by gas chromatography) were purchased from Tokyo Kasei Kogyo Co., Tokyo. Benzyl alcohol, acetaldehyde (80%) and benzaldehyde were purchased from Koso Chemical Co., Tokyo. No significant amount of impurity was observed in these reagents by gas chromatography.

RESULTS AND DISCUSSION

I. Extraction of ADH

Each 0.2 g of strawberry seeds were homogenized in mortar with 2 ml of 0.1 M buffer at various pH (for buffers used, see Fig. 1.) containing 1 mM reduced glutathione and 2% polyvinylpyrrolidone (M. W.=40,000). After centrifugation for 30 min at 13,000 $\times g$ at 0°C, the supernatant was filtered through a glass filter funnel (3G3) to remove floating particles. The filtrate was designated "crude extract."

The crude extracts prepared with various buffers (pH 4.5~10.0) were assayed activities for oxidation of ethanol, allyl alcohol, benzyl

FIG. 1. Extraction of ADHs with Various Buffer.

Following buffers containing 1 mM of reduced glutathione and 2% of polyvinylpyrrolidone were used: 0.1 M citric acid-0.2 M Na_2PO_4 (McIlvaine) buffer, pH 4.5, 6.0 and 7.0; 0.1 M sodium-potassium phosphate buffer, pH 6.0 and 7.0; 0.1 M glycine-NaOH buffer, pH 8.5 and 10.0.

○, ethanol: NAD; □, allyl alcohol: NAD; ▲, benzyl alcohol: NADP; ◆, geraniol: NADP.

alcohol and geraniol at their optimum pH (see effect of pH on enzyme activity), Fig. 1.

ADH which reacted with ethanol and allyl alcohol needed NAD as cofactor, and NADP could not replace NAD through the experiment. In contrast, ADH which reacted with benzyl alcohol and geraniol needed NADP as cofactor, and NAD could not replace NADP.

The maximum stability of NAD specific ADH and NADP specific ADH were both found at pH 7.0. The stability during extraction at higher pH, however, displayed a big difference between them. As is apparent from Fig. 1, NADP-dependent ADH was considerably stable at pH 10.0, while NAD-dependent ADH lost almost of all activity at the same pH.

Strawberry seeds ADH was found to be rather labile at lower pH. In the comparison with the extraction performed at pH 7.0, NAD specific ADH and NADP specific ADH lost their activity around 30~40% during the extraction at pH 6.0. ADH activity disappeared completely when the crude extract was prepared with pH 4.5 buffer. Therefore, the

control of pH is critical problem for the extraction of strawberry seeds ADH.

All procedures were carried out at 4°C unless otherwise noted.

II. Ammonium sulfate fractionation

Five grams of strawberry seeds were homogenized with sodium-potassium phosphate buffer (0.1 M, pH 7.0) according to the procedure mentioned in extraction of ADH. The crude extract was fractionated by precipitation with solid ammonium sulfate. Fractions corresponding to 0~35%, 35~50% and 50~70% saturation were prepared by centrifugation at $14,000 \times g$ for 30 min at 0°C. Then the precipitates were dissolved in 0.05 M sodium-potassium phosphate buffer (pH 7.0) containing 1 mM reduced glutathione and dialyzed against the same buffer. The dialyzed protein solution was centrifuged at $10,000 \times g$ for 30 min to obtain clear solution.

Each fraction was assayed ADH activity for ethanol, allyl alcohol, benzyl alcohol and geraniol.

NAD-dependent ADH and NADP-dependent ADH were both concentrated in a protein fraction precipitated between 35 and 50% ammonium sulfate saturation (Table I). Specific activity of NAD-dependent ADH was raised 2.5~2.7 folds, and that of NADP-dependent ADH was raised 2.8~3.0 folds of crude extract, respectively. However, the difference was observed in the fraction of 50~70% ammonium sulfate saturation. NADP-

dependent ADH was concentrated approximately 1.8 folds in this fraction, whereas NAD-dependent ADH was not concentrated.

III. Disc electrophoresis

The different behavior between NAD-dependent ADH and NADP-dependent ADH in ammonium sulfate fractionation indicates that these activities result from different proteins. To obtain further knowledge of this matter, disc electrophoresis was performed.

The sample enzyme solution was prepared from 10 g of strawberry seeds by extraction with 0.1 M sodium-potassium phosphate buffer (pH 7.0) followed by ammonium sulfate fractionation (25~65% saturation). The protein fraction was dissolved with 2 ml of 0.05 M sodium-potassium phosphate buffer (pH 7.0), and then dialyzed against same buffer (500 ml $\times$ 3). After addition of sucrose (40% at final concentration), 0.2 ml of sample was layered on the top of the gel.

The results obtained from disc electrophoresis were (1) ethanol oxidizing enzyme showed same migration profile as allyl alcohol oxidizing enzyme, (2) benzyl alcohol oxidizing enzyme indicated similar migration profile as geraniol oxidizing enzyme, and (3) the mobility of NAD-dependent ADH was smaller than that of NADP-dependent ADH, Fig. 2.

TABLE I. AMMONIUM SULFATE FRACTIONATION

	Crude extract	Ammonium sulfate fractionation			
		0~35%	35~50%	50~70%	
Ethanol: NAD	7.544 (0.199)	0.142 (0.006)	3.224 (0.496)	1.592 (0.167)	
Allyl alcohol: NAD	8.866 (0.233)	0.202 (0.009)	4.030 (0.620)	1.793 (0.188)	
Benzyl alcohol: NADP	1.370 (0.036)	0.101 (0.005)	0.645 (0.099)	0.645 (0.068)	
Geraniol: NADP	0.645 (0.017)	0.040 (0.002)	0.343 (0.052)	0.282 (0.030)	

(): Specific activity (unit/mg protein)

FIG. 2. Polyacrylamide Gel Disc Electrophoresis of Strawberry Seeds ADHs.

The protein moved from the cathode to the anode.

A; Ethanol: NAD, B; Allyl alcohol: NAD, C; Benzyl alcohol: NADP, D; Geraniol: NADP

The staining for enzyme activity is described in the methodology section.

The result (1) revealed that ethanol and allyl alcohol oxidizing activities resulted from the identical enzyme. This is quite understandable because ADH has broad substrate specificity in general, and some ADHs which react with many aliphatic alcohols (*e.g.* ethanol, propanol, allyl alcohol, *trans*-2-hexenol) belong to those called aliphatic alcohol dehydrogenases. NAD-dependent strawberry seeds ADH which reacts with ethanol and allyl alcohol is thought to belong to aliphatic alcohol dehydrogenase.

The result (2) indicated that benzyl alcohol oxidizing activity was caused by the same enzyme as geraniol oxidizing one. NADP-dependent ADH activity was existed at wide range; it seemed that two or three isozymes were contained, though further investigations were required for the evidence.

The result (3) showed that strawberry seeds contained at least two ADHs; one is NAD specific and reacts with ethanol and allyl alcohol, and the other NADP specific and reacts with benzyl alcohol and geraniol.

IV. Identification of products

Direct analysis of the reaction mixture containing substrate, cofactor and enzyme from strawberry seeds in glycine-NaOH buffer was carried out by gas chromatography. We observed that ethanol was oxidized to acetaldehyde, and that benzyl alcohol was oxidized to benzaldehyde. From the reaction mixture of geraniol, NADP and enzyme, however, we found two aldehyde, geometric isomers; geranial and neral. As geraniol used was nerol free, *trans* to *cis*-isomerization was accompanied in the oxidation of geraniol.

V. Properties of ADH

1) *Effect of pH on enzyme activity.* Effect of pH on strawberry seeds ADHs was examined using following buffer systems: 0.1 M citric acid-0.2 M Na₂PO₄ (McIlvaine) buffer for pH 3.0~pH 8.0; 0.1 M Tris-HCl buffer for pH 7.2~pH 8.5; 0.1 M glycine-NaOH buffer for pH 8.5~pH 11.0 (Fig. 3).

The optimum reaction pH of NAD-dependent ADH was found at pH 9.0, while that

FIG. 3. Effect of pH on Enzyme Activity.

○, ethanol: NAD; ●, ethanol: NADP; □, allyl alcohol: NAD; ■, allyl alcohol: NADP; △, benzyl alcohol: NAD; ▲, benzyl alcohol: NADP; ◇, geraniol: NAD; ◆, geraniol: NADP.

of NAD-dependent ADH was found at pH 9.5. Neither NAD-dependent ADH activity nor NADP-dependent ADH activity was observed at the pH range below 6.0. The alternation of NAD with NADP made the enzymes inactive through the experiments.

Though it is omitted from Fig. 3, the enzymes did not react with cyclohexanol under any conditions employed.

2) *Thermostability.* The difference between NAD-dependent ADH and NADP-dependent ADH was also observed in thermostability as found in the effect of pH.

Strawberry seeds ADHs were relatively stable to heat-treatment. NAD-dependent ADH activity and NADP-dependent ADH activity were both retained after heating at 45°C for 15 min. Although NADP-dependent ADH lost the activity completely after heat-treatment at 60°C, NAD-dependent ADH activity was retained approximately 30% at the same conditions (Fig. 4).

3) *Effect of freezing.* NAD-dependent ADH in crude extract lost the activity rapidly to about 20% in 24 hr when it was kept at 5°C, while 95% of NADP-dependent ADH activity was remained at the same conditions.

FIG. 4. Effect of Temperature on Enzyme Stability. O, ethanol: NAD; □, allyl alcohol: NAD; ▲, benzyl alcohol: NADP; ◆, geraniol: NADP.

The freezing (-20°C) of crude extract gave the desirable effect on NAD-dependent ADH; 60% of activity was remained in 24 hr. NADP-dependent ADH was more stable than NAD-dependent ADH under these conditions.

Ammonium sulfate fractionation (25~65% saturation) improved the stability considerably; 75% of NAD-dependent ADH and NADP-dependent ADH activity were remained for 30 days (-20°C). Strawberry seeds ADHs after ammonium sulfate fractionation were quite stable at least 30 days in the presence of 25% sucrose at -20°C , Fig. 5.

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FIG. 5. Effect of Freezing on Enzyme Stability.

--, crude extract; 5°C ; —, crude extract; frozen (-20°C); ---, after ammonium sulfate fractionation (25~65% saturation); frozen (-20°C); -----, after ammonium sulfate fractionation (25~65% saturation) + 25% sucrose; frozen (-20°C). O, ethanol: NAD; □, allyl alcohol: NAD; ▲, benzyl alcohol: NADP; ◆, geraniol: NADP.

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