

## Alcohol Formation from Aldehydes by Endogeneous Alcohol Dehydrogenase in Soybean Extracts

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*n*-Hexanal (a major component of beany flavor) can be reduced to *n*-hexanol by endogenous alcohol dehydrogenase in a soybean extract. The substrate specificity for other aldehydes and the participation of coenzymes (NADH and NAD<sup>+</sup>) in this reaction were investigated. A soybean extract was incubated with various aliphatic aldehydes (carbon number 3-9) at 37°C, and the amount of alcohols formed was determined. The enzyme had a wide substrate specificity, the enzyme activity decreasing as the carbon number of the aldehydes increased (carbon number 3>4>5>6>7>9>8). The enzyme activity was markedly promoted by the addition of NAD<sup>+</sup> as well as NADH. When a soybean extract was incubated with NAD<sup>+</sup>, NADH was formed, but no NADH was formed in the soybean extract after heating at 100°C for 5 min. These results suggest that NAD<sup>+</sup> was converted to NADH by the action of an enzyme(s) in the soybean extract.

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### INTRODUCTION

Soybeans have long been established in Japan as a major source of nutrition and are processed into foods such as *tofu*, *natto* and *miso*. Soy proteins are high in nutritional quality<sup>1)</sup> and functionality.<sup>2)</sup> In addition, soy proteins are effective for minimizing the plasma cholesterol level,<sup>3)-5)</sup> and soybean foods have recently become of greater interest throughout the world. However, the characteristic grassy, beany and green flavors of soybean formed during processing and cooking have hindered its wide utilization. The major contributors to these flavors are the volatile carbonyl compounds which are enzymatically derived from the hydroperoxides of unsaturated fatty acids.<sup>6)</sup> Soybean contains three lipoxygenase isozymes (L-1, L-2 and L-3) which exhibit different kinetic behavior.<sup>7)-9)</sup> We have previously demonstrated the mechanism for the formation of *n*-hexanal, one of the major compounds of soybean flavor, as follows: In a

soybean homogenate, in which the L-2 isozyme reacts predominately with linoleic acid to produce 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-hydroperoxide of linoleic acid), which is cleaved by hydroperoxide lyase to produce *n*-hexanal under normal pH conditions (pH 6-7).<sup>9)10)</sup>

Various methods have been reported to remove the soybean flavors: limited proteolysis,<sup>11)</sup> extraction with organic solvents,<sup>12)</sup> oxidation by aldehyde dehydrogenase<sup>13)14)</sup> and oxidation by *Acetobacter aceti*.<sup>15)</sup> Hamilton has reported that the threshold value for *n*-hexanal was about one tenth that of *n*-hexanol.<sup>16)</sup> Therefore, the conversion of *n*-hexanal to *n*-hexanol may be useful for reducing the intensity of the soybean flavors. Alcohol dehydrogenase has been used to remove aldehydes from milk,<sup>17)</sup> orange juice<sup>18)</sup> and fish protein hydrolysate.<sup>19)</sup> A method for removing the aldehydes in soymilk by the alcohol dehydrogenase of *Aspergillus oryzae* has also been reported by Yamaguchi *et al.*<sup>20)</sup> In the previous paper, we reported that *n*-hexanal was reduced to *n*-hexanol by endogenous alcohol dehydrogenase in a soybean extract under

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alkaline conditions.<sup>21)</sup> The application of this endogenous enzyme would be more convenient for removing aldehydes than other methods, although its action has not yet been completely clarified. Furthermore, the substrate specificity against other aldehydes has not been fully elucidated. This study aims elucidate the substrate specificity and the participation of coenzymes NADH and NAD<sup>+</sup> in this enzyme reaction.

## MATERIALS AND METHODS

### 1) Materials

Soybean (*Glycine max*, var. Tsuru-no-ko) was obtained from Mizuno Seedling Co. (Kyoto). Various aldehydes and alcohols were purchased from Nacalai Tesque Inc. (Kyoto) and Wako Pure Chemical Industries Ltd. (Osaka). NADH and NAD<sup>+</sup> were purchased from Kojin Co. Ltd. (Tokyo), and linoleic acid (>99% purity) was purchased from Wako. All other chemicals were purchased from Nacalai and Wako.

### 2) Preparation of the linoleic acid solution

A linoleic acid solution (0.4%, pH 6.7) containing 0.4% Tween 20 was prepared according to the procedure of Grossman and Zakut.<sup>22)</sup>

### 3) Preparation of the soybean extracts

Soybean seeds (5 g) were soaked overnight in water at 4°C. After removing the seed coat, the soaked seed was homogenized in 80 ml of 0.05 M phosphate buffer at pH 8.0 with a glass homogenizer (Potter-Elvehjem type) in an ice bath. The resulting homogenate was centrifuged at 8,000 rpm and 4°C for 20 min. The supernatant was used for the experiments shown in Figs. 1 and 2 (the whole seed extract).

Soybean flour (20 g) defatted with *n*-hexane was suspended in 300 ml of 0.05 M phosphate buffer at pH 6.7 or 8.0 and 4°C for 1 hr with stirring. The resulting suspension was then centrifuged at 8,000 rpm and 4°C for 20 min, the supernatant being used for the experiments shown in Fig. 3, and Tables 1 and 2 (the defatted flour extract).

### 4) Determination of alcohols

The soybean extracts were incubated with aldehydes under the conditions described in the figure legends. After incubating, the resulting alcohols were extracted by shaking with an ethyl ether and saturated NaCl solution at room temperature for 5 min. After centrifuging at 2,500 rpm and 5°C for 10 min, the organic layer was flash-evaporated

to 1 ml with nitrogen gas. The resulting concentrate was used for a gas-liquid chromatographic (GLC) analysis, which was carried out on a Shimadzu GC-9A gas chromatograph equipped with a flame ionization detector and a glass column (3 mm × 2 m) packed with 10% PEG 20M on Chromosorb W AW DMCS (80–100 mesh). The flow rate of nitrogen was 50 ml/min, the column oven temperature being programmed at 80°C for first 4 min, from 80 to 180°C at 15°C/min, and then 180°C for 10 min. The alcohols used as the internal standards were as follows: *n*-butanol (C<sub>4</sub>) for determining *n*-propanol (C<sub>3</sub>), *n*-pentanol (C<sub>5</sub>) for *n*-butanol, *n*-hexanol (C<sub>6</sub>) for *n*-pentanol, *n*-heptanol (C<sub>7</sub>) for *n*-hexanol and *n*-octanol (C<sub>8</sub>), and *n*-octanol for *n*-heptanol and *n*-nonanol (C<sub>9</sub>). The alcohol-forming activity is expressed as the alcohol-forming level (nmol) per mg of protein.

### 5) Determination of *n*-hexanal

*n*-Hexanal was determined by the same method as that used for the alcohols, *n*-heptanol being used as the internal standard.

### 6) Identification of alcohols

The alcohols were identified with a Hitachi gas chromatograph-mass spectrometer (M-80), using the same sample as that prepared for GLC analysis. The column and oven temperature program was the same as those for GLC analysis.

### 7) Protein content

Protein content was determined according to the procedure of Lowry *et al.*<sup>23)</sup> with bovine serum albumin as the standard.

### 8) Determination of NADH

The defatted seed extract (4 mg protein/ml at pH 8.0) was incubated in the presence of 1 mM NAD<sup>+</sup> at 37°C for 30 min. The protein was then removed by hot 70% ethanol according to the procedure of Okuda.<sup>24)</sup> After centrifuging at 3,000 rpm and 5°C for 15 min, the supernatant was evaporated under reduced pressure. The resulting residue was dissolved in a 25 mM Tris-HCl buffer (pH 8.7) for a high-performance liquid chromatographic (HPLC) analysis according to the procedure of Aso *et al.*<sup>25)</sup> A Shimadzu LC-6A liquid chromatograph equipped with a UV detector (SPD-6A) was used for this analysis. The detector was set at 260 nm, and a Lichrosorb RP-18 column (ODS type, 5 μm, 4 × 150 mm) was used. A C-R3A integrator (Shimadzu) was used for calculating the peak areas. The mobile phase was a

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0.15 M sodium phosphate buffer (pH 6.8) containing 1 mM EDTA at a flow rate of 1.5 ml/min, and the temperature of the column was 25–30°C.

## RESULTS AND DISCUSSION

1) *n*-Hexanol formation with a linoleic acid substrate

The optimum pH for alcohol dehydrogenase (of yeast,<sup>26)</sup> horse liver,<sup>27)</sup> and tea seed<sup>28)</sup>) has been reported to be 7–9. We have previously reported that its optimum pH in a soybean extract was 8–9, and that the *n*-hexanol-forming activity at pH 6.7 was about half of that at pH 8.0 when the soybean extract was incubated with *n*-hexanal added exogenously.<sup>21)</sup> We also reported that *n*-hexanal did not apparently decrease when the soybean extract was incubated at pH 6.7.<sup>21)</sup> It is probable that the *n*-hexanal-forming activity from linoleic acid is much higher than its reduction. However, it was not clear how much *n*-hexanol would be formed from linoleic acid *via* *n*-hexanal under normal pH conditions (pH 6–7) in a soybean extract. In this study, *n*-hexanol was determined at pH 6.7 in the soybean extract model system containing defatted flour and linoleic acid (Table 1). *n*-Hexanal and *n*-hexanol were formed from linoleic acid. The concentration of *n*-hexanol formed in the period from 30 to 60 min was lower than that during the initial 30 min, although sufficient *n*-hexanal was formed as the substrate of alcohol dehydrogenase. Grosch has reported that, in pea extracts, linoleic acid hydroperoxide formed from linoleic acid by lipoxygenase oxidized the active site (SH group) of alcohol dehydrogenase to inactivate the enzyme.<sup>29)</sup> This may also be the case in a soybean extract.

Table 1. Formation of *n*-hexanal and *n*-hexanol from exogenous linoleic acid in a defatted soy flour extract at 37°C

Incubation time (min)	<i>n</i> -Hexanal (nmol/mg of protein)	<i>n</i> -Hexanol (nmol/mg of protein)
Before incubation	0.59	0.19
30	1.22	0.30
60	1.39	0.35

A defatted soy flour extract (9 ml, 20 mg of protein/ml, pH 6.7) containing 2.8 mM linoleic acid was incubated at 37°C.

## 2) Substrate specificity of alcohol dehydrogenase

The substrate specificity of alcohol dehydrogenase was determined as shown in Fig. 1. The whole seed extract was incubated with various aldehydes, and the concentrations of alcohols formed were determined by GLC analysis. All exogenous aliphatic aldehydes (carbon number 3–9) were reduced to the corresponding alcohols, and no other products were detected by the GLC analysis. The alcohol dehydrogenase of soybean had wide substrate specificity for various aliphatic aldehydes, as has been observed in other higher plants.<sup>29)–31)</sup> Each alcohol increased during incubation (Fig. 1), the alcohol-forming activity of alcohol dehydrogenase increasing as the carbon number of the aldehydes decreased. This observation is also consistent with the case for pea alcohol dehydrogenase reported by Grosch.<sup>29)</sup>

## 3) Effect of coenzyme on alcohol formation in a soybean extract

Alcohol dehydrogenase in soybean catalyzes the reducing reaction in the presence of NADH.<sup>21)</sup> The alcohol-forming activity was determined in the presence of exogenous NADH (Fig. 2). The

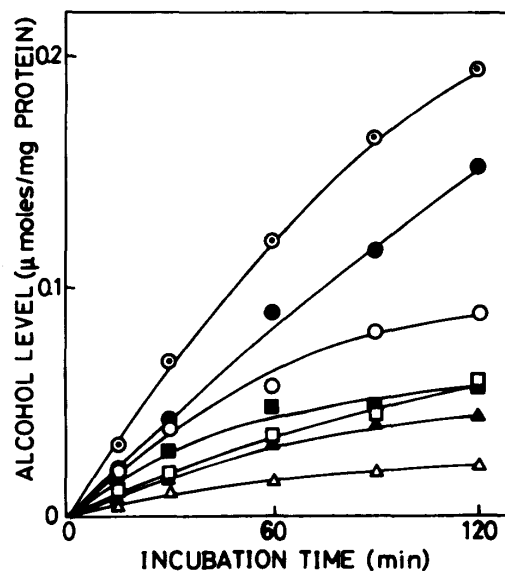


Fig. 1. Time-course for alcohol formation from various aldehydes in whole soybean seed extracts

Whole seed extracts (3 ml, 17 mg of protein/ml, pH 8.0) containing 20 mM of various aldehydes (C<sub>3</sub>–C<sub>9</sub>) were incubated at 37°C. ⊙, *n*-propanol; ●, *n*-butanol; ○, *n*-pentanol; □, *n*-hexanol; ■, *n*-heptanol; △, *n*-octanol; ▲, *n*-nonanol.

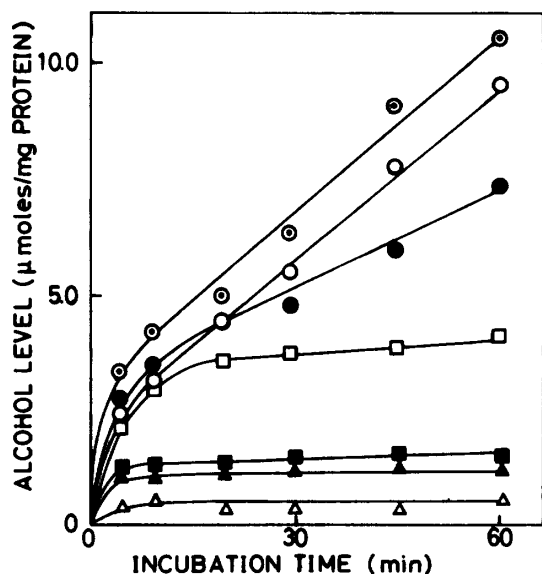


Fig. 2. Time-course for alcohol formation from various aldehydes in whole soybean seed extracts in the presence of exogenous NADH

Whole seed extracts (3 ml, 0.4 mg of protein/ml, pH 8.0) containing 20 mM of various aldehydes ( $C_3$ – $C_9$ ) and 1 mM NADH were incubated at 37°C.  $\odot$ , *n*-propanol;  $\bullet$ , *n*-butanol;  $\circ$ , *n*-pentanol;  $\square$ , *n*-hexanol;  $\blacksquare$ , *n*-heptanol;  $\triangle$ , *n*-octanol;  $\blacktriangle$ , *n*-nonanol.

enzyme activity was markedly increased compared with the case in the absence of exogenous NADH (Fig. 2), suggesting that the soybean extract did not contain enough endogenous coenzyme for full enzyme activity. However, we have previously reported that *n*-hexanol formation from *n*-hexanal was strongly promoted in the presence of exogenous NAD<sup>+</sup> when compared with the case for the absence of NAD<sup>+</sup>.<sup>21)</sup> When NAD<sup>+</sup> was added to the soybean extract, the formation of each alcohol from the corresponding aldehyde was increased, but the level was lower than that in the case of NADH (data not shown). This observation suggests that NAD<sup>+</sup> was converted to NADH by another reaction mechanism in the soybean extracts.

#### 4) Determination of NADH

To confirm that NAD<sup>+</sup> was converted to NADH by another reaction mechanism, the defatted seed extract was incubated with NAD<sup>+</sup>, and the level of NADH formed was determined by HPLC. No NADH was detected in the defatted seed extract with or without NAD<sup>+</sup> before incubation (Fig. 3A), but NADH was detected after 30 min of incubation (Fig. 3B). The level of NADH was

about 60  $\mu$ M after 15 or 30 min of incubation (Table 2). The apparent  $K_m$  value of NADH for the reduction of *n*-hexanal by the alcohol dehydrogenase was previously reported as 70  $\mu$ M in a soybean extract.<sup>21)</sup> Hence, the concentration of NAD<sup>+</sup> formed from NADH seems to have been enough for the enzyme to reduce the aldehyde. On the other hand, when the soybean extract was heated at 100°C for 5 min, no NADH was detected in the extract after incubating with NAD<sup>+</sup> for 30 min (Table 2). This result indicates that the conversion of NAD<sup>+</sup> to NADH was caused by the action of an enzyme(s). Moreover, a reducing compound seems to have participated in this mechanism.

Figure 4 indicates the reaction system which

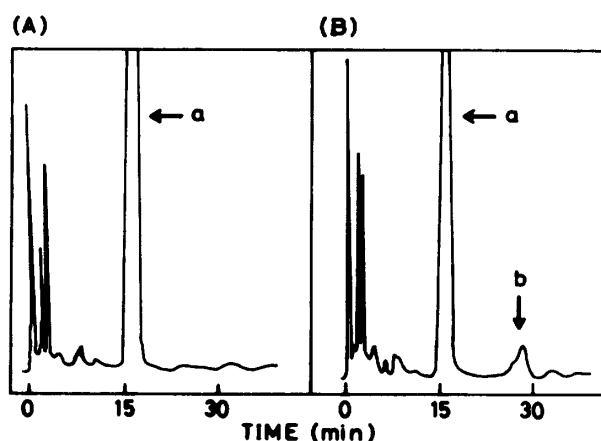


Fig. 3. HPLC chromatogram of the reaction products after incubating a defatted soy flour extract with exogenous NAD<sup>+</sup>

The defatted flour extract (1.5 ml, 5 mg of protein/ml, pH 8.0) containing 1 mM NAD<sup>+</sup> was incubated at 37°C. Peak a, NAD<sup>+</sup>; peak b, NADH. (A): before incubating, (B): after incubating at 37°C for 30 min.

Table 2. Formation of NADH from NAD<sup>+</sup> in a defatted soy flour extract at 37°C

Incubation time (min)	NADH ( $\mu$ M)	
	Not heated	Heated*
5	22	—
15	62	—
30	58	0

A defatted soy flour extract (1.5 ml, 5 mg of protein/ml, pH 8.0) containing 1 mM NAD<sup>+</sup> was incubated at 37°C. \* Defatted flour extract was heated at 100°C for 5 min.

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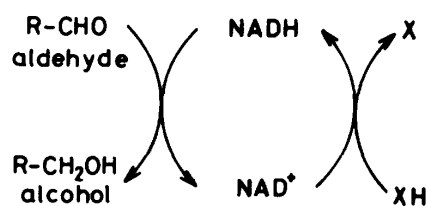


Fig. 4. Possible mechanism for the reduction of aldehydes to alcohols in a whole soybean extract in the presence of NAD<sup>+</sup>

XH, reducing compound; X, oxidized compound.

converts aldehydes to alcohols that follows from these observations. After dialyzing the extracts to remove low molecular compounds, the activity was markedly lower in the presence of NAD<sup>+</sup> than in the presence of NADH.<sup>21)</sup> Therefore, the reducing compound (XH) seems to have been a low molecular compound, and its identification is now in progress.

Our present results demonstrate that the endogenous alcohol dehydrogenase and exogenous co-enzymes converted aldehydes to alcohols in a soybean extract model system. This reaction could be applicable to soybean foods to reduce the intensity of the beany flavor.

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大豆抽出液における内因性アルコールデヒドロゲナーゼ  
によるアルデヒドからアルコールの生成

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代表的な豆臭成分である *n*-ヘキサナールは, 大豆抽出液中においてはアルコールデヒドロゲナーゼの作用により *n*-ヘキサノールに還元される。本研究では本酵素反応の他のアルデヒドに対する基質特異性及び本反応に対する補酵素 (NADH, NAD<sup>+</sup>) の作用機構について検討した。大豆抽出液に種々の脂肪族アルデヒド (炭素数 3~9) を添加し 37°C でインキュベートし, 生成したアルコールを定量した。本酵素反応の基質特異性は広く, 各アルデヒドに対する相対活性は, アルデヒドの炭素数が増えるにつれ減少した (炭素数 3 > 4 > 5 > 6 > 7 > 9 > 8)。また, これらの反応は NADH のみならず, NAD<sup>+</sup> 添加によっても促進された。大豆抽出液と NAD<sup>+</sup> をインキュベートすると, NADH は生成したが, 加熱処理 (100°C, 5 分間) した大豆抽出液では, NADH の生成はみられなかった。このことから, 大豆抽出液中には NAD<sup>+</sup> を NADH に変換する酵素系が存在することが示唆された。

キーワード: 大豆, 豆乳, 豆臭, *n*-ヘキサナール, アルデヒド, アルコールデヒドロゲナーゼ。