Note

Purification and Characterization of NAD-dependent Alcohol Dehydrogenase and NADH-dependent 2-Oxoaldehyde Reductase from Parsley

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The Maillard reaction under physiological conditions results in a loss of biological activity, and has been proposed as a cause of aging in cells and tissues.¹⁾ 3-Deoxyglucosone (3DG), a 2-oxoaldehyde compound, is a major intermediate in the Maillard reaction.^{2,3)} Therefore, 3DG-metabolizing enzymes may possibly prevent the Maillard reaction by the reduction or oxidation of 3DG. It has been reported that 3DG was metabolized by NAD(P)-dependent 2-oxoaldehyde dehydrogenases of rat and sheep livers, but not by glyoxalase.⁴⁾ We have found that NAD(P)H-dependent 2-oxoaldehyde reductases of porcine liver also metabolized 3DG.^{5,6)}

However, there has been no report on the 2-oxo-

aldehyde-metabolizing enzyme in plants. Therefore, we searched for the presence of 2-oxoaldehyde-metabolizing enzymes in some vegetables, and reported the isolation of an NADPH-dependent 2-oxoaldehyde reductase, which catalyzes the reduction 3DG to 3-deoxyfructose, from parsley in our previous papers.^{7,8)} The present paper describes how an NAD-dependent alcohol dehydrogenase was purified and characterized, and how an NADH-dependent 2-oxoaldehyde reductase was partially purified and characterized from parsley leaves. The activity of these two enzymes towards 3DG is discussed.

Enzyme activity was measured by the rate of decrease in absorbance at 340 nm of NADH ($\epsilon_{340} = 6220$). The assay mixture contained, in a total volume of 1 ml, 100 mM Na-phosphate buffer (pH 7.0), 0.3 mM NADH, 10 mM acetaldehyde, methylglyoxal or 3DG, and a suitable amount of the enzyme. One activity unit is defined as that reducing 1 mmol of NADH per min. 3DG was prepared as described in our previous paper.⁷⁾ Protein concentration was determined by the method of Lowry *et al.*,⁹⁾ using bovine serum albumin as the standard.

Fresh commercial parsley leaves (125 g) were homogenized in a Waring blender at 4°C for 2 min with 250 ml of 20 mM sodium phosphate buffer (pH 7.2) containing 10 mM 2-mercaptoethanol (buffer A). The suspension was squeezed through gauze and centrifuged. After the supernatant had been dialyzed against buffer A, the crude extract was obtained. The crude extract was fractionated into three fractions by ammonium sulfate, and the main enzymatic activity was found in the 40–65% ammonium sulfate fraction. After dialyzing against buffer A, the main activity fraction was placed in a DEAE-cellulose (Whatman DE-52) column (1.8 × 30 cm) that had been previously equilibrated with buffer A, and eluted with a

Fig. 1. Elution Pattern of Enzyme I and Enzyme II from a DEAE-Cellulose Column.

The conditions for column chromatography are described in the text. (—●—), absorbance at 280 nm; (—■—), enzymatic activity towards methylglyoxal; (---), NaCl gradient of the eluate from 0 to 260 mM. Fractions of 6 ml were collected.

Table I. PURIFICATION OF NAD-DEPENDENT ALCOHOL DEHYDROGENASE

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery of activity (%)	Purity (-fold)
Crude extract	425.4	62.5	0.147	100	1
(NH ₄) ₂ SO ₄ ppt	124.2	52.3	0.421	83.7	2.9
DEAE-cellulose	12.3	34.7	2.821	55.5	19.2
Hydroxyapatite	2.5	21.1	8.440	33.8	57.4
NAD-agarose	0.6	10.3	17.167	16.5	116.8

Enzymatic activity was toward methylglyoxal.

linear concentration gradient of NaCl from 0 to 260 mM in buffer A. As shown in Fig. 1, two distinct peaks of activity were observed. The major activity peak (enzyme I) was eluted at 50 mM NaCl concentration, and the minor one (enzyme II) at 200 mM NaCl concentration. The active fractions of enzyme I and enzyme II were each dialyzed against buffer A and concentrated by ultrafiltration. Methylglyoxal was used as a substrate at different stages of the enzymatic purification.

Further purification of enzyme I. The enzyme I fractions were placed in a hydroxyapatite column (1 × 15 cm) equilibrated with buffer A and eluted with a linear concentration gradient from 20 to 400 mM of K-phosphate (pH 7.2) containing 10 mM 2-mercaptoethanol. Enzymatic activity was eluted at about 260 mM K-phosphate. The active fractions were dialyzed against buffer A, concentrated by ultrafiltration and then placed in a column (1 × 5 cm) of NAD-agarose (Sigma) equilibrated with buffer A. The column was washed with buffer A, and then eluted with buffer A containing 1.5 mM NAD. The active fractions were collected and dialyzed against buffer A.

Table I summarizes the purification process for enzyme I. The fraction from the final purification step had a single band of protein on SDS-polyacrylamide gel electrophoresis (data not shown).

Further purification of enzyme II. The foregoing enzyme II fractions from the DEAE-cellulose column were placed in a hydroxyapatite column (1 × 15 cm) equilibrated with buffer A and eluted with a linear concentration gradient from 20 to 400 mM of K-phosphate (pH 7.2) containing 10 mM 2-mercaptoethanol. The active fractions were pooled and concentrated by ultrafiltration, and then applied to a Sephadex G-100 column (2 × 85 cm) equilibrated with buffer A containing 0.1 M NaCl, eluting with the same buffer. The enzyme fractions were collected and dialyzed against buffer A. After concentrating, the enzyme was placed in a column (1 × 10 cm) of blue Sepharose CL-6B (Sigma) equilibrated with buffer A. The enzyme was eluted with buffer A and a linear concentration gradient from 20 to 1000 mM of K-phosphate (pH 7.2) containing 10 mM 2-mercaptoethanol. The enzyme fractions, which were

Table II. SUBSTRATE SPECIFICITY OF NAD-DEPENDENT ALCOHOL DEHYDROGENASE (I) AND NADH-DEPENDENT 2-OXOALDEHYDE REDUCTASE (II)

Substrate	Relative activity (%)	
	I	II
Acetaldehyde	100.0	2.6
Propanal	94.0	—
Methylglyoxal	11.9	29.5
2,3-Pentanedione	5.5	—
Phenylglyoxal	1.1	57.7
Benzaldehyde	1.1	2.6
Hydroxyacetone	0.2	0
Glyceraldehyde	0.1	15.4
3-Deoxyglucosone	0.1	100.0
Dihydroxyacetone	0.1	5.1
Diacetyl	0	—
Glucosone	—	59.0
Glyoxal	—	10.3
Fructose	0	0
Glucose	0	0

The enzyme was assayed with 10 mM of different substrates in 100 mM Na-phosphate buffer (pH 7.0) containing 0.3 mM NADH at 25°C. —, not determined.

eluted at about 540 mM K-phosphate, were pooled and dialyzed against buffer A. Since it was very difficult to completely achieve, no further attempt was made to purify the enzyme.

Characterization of enzyme I and enzyme II. The molecular weight of purified enzyme I was estimated as 68,000 in a column of Sephadex G-100, and showed a single band of 37,000 on SDS-polyacrylamide gel electrophoresis. Therefore, this enzyme was likely to have contained two subunits of equal molecular weight. The optimum pH in 100 mM sodium phosphate buffer was 5.7. The *K_m* and *V_{max}* values for the acetaldehyde were 0.6 mM and 279.4 units/mg, respectively. The enzyme activity was not affected by EDTA, but was inhibited almost completely

by 0.1 mM *p*-chloromercuribenzoate. This enzyme also had alcohol dehydrogenase activity with NAD and ethanol as the substrate. The optimum pH for the dehydrogenase activity was 8.9. Table II shows the substrate specificity of the enzyme. This enzyme showed very high activity for aliphatic aldehyde, and also showed considerable activity towards methylglyoxal, although the *K_m* value (35 mM) was higher than to acetaldehyde. The enzyme had very low activity towards 3DG. The enzyme specifically required NADH as a coenzyme and had only 1.6% activity when NADPH was substituted for NADH. These properties just described suggest that purified enzyme I corresponds to NAD-dependent alcohol dehydrogenase (EC 1.1.1.1).¹⁰⁾ Parsley leaves had very high activity for NAD-dependent alcohol dehydrogenase, the enzyme activity being 8.1×10^{-3} units/g of tissue towards the acetaldehyde.

On the other hand, the optimum pH of partially purified enzyme II was 7.0 in 100 mM Na-phosphate buffer. The *K_m* value for 3DG was 5 mM. The enzyme activity was not inhibited by EDTA, but was lost by 0.1 mM *p*-chloromercuribenzoate. The substrate specificity is shown in Table II, this enzyme having high activity with 2-oxoaldehydes such as 3DG, glucosone, phenylglyoxal, and methylglyoxal. 3DG was the best substrate among the compounds tested. The enzyme also showed considerable activity towards glyceraldehyde, glyoxal and dihydroxyacetone. When NADH was replaced by NADPH, the enzyme had only 3.8% of the activity shown in the presence of NADH. According to the properties just described, we tentatively designated enzyme II to be NADH-dependent 2-oxoaldehyde reductase. The enzyme activity (1×10^{-5}

units/g of tissue) was markedly lower than the NADPH-dependent one (1.74×10^{-4} units/g of tissue) in parsley leaves.⁸⁾ This parsley NADH-dependent 2-oxoaldehyde reductase could be separated from the NADPH-dependent one of parsley on a DEAE-cellulose column, the former being eluted at a higher salt concentration than the latter in the DEAE-cellulose column. Moreover, the NADH-dependent one required NADH as a coenzyme for activity, while the NADPH-dependent one had no activity with NADH.⁷⁾

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