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PURIFICATION AND PROPERTIES OF ALDEHYDE DEHYDROGENASE FROM *PROTEUS VULGARIS*

YASUTAKE SUGAWARA * and SHOJI SASAKI **

Department of Botany, Faculty of Science, Hokkaido University, Sapporo (Japan)

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Summary

NADP-linked aldehyde dehydrogenase (aldehyde : $NADP^+$ oxidoreductase, EC 1.2.1.4) was purified from *Proteus vulgaris* to the stage of homogeneity as judged by ultracentrifugation and polyacrylamide gel electrophoresis. The molecular weight of the purified enzyme was estimated to be 130 000 by gel filtration.

The enzyme which was crystallized from ammonium sulfate solution, lost its activity.

The enzyme did not require coenzyme A, and the reaction was completely dependent on ammonium ions which could be partially replaced by Rb^+ or K^+. The optimum pH was about 9. Broad substrate specificity was observed and K_m values for propionaldehyde, acetaldehyde and isovaleraldehyde were $1.7 \cdot 10^{-5}$, $4 \cdot 10^{-5}$ and $3 \cdot 10^{-5}$ M, respectively.

The physiological role of the enzyme in living cells is obscure, but might account for another degradative pathway of L-leucine in *P. vulgaris* differing from the established pathway.

Introduction

Previously we reported that a decarboxylase operating in the degradative pathway of L-leucine in *Proteus vulgaris* catalyzed the decarboxylation of isocaproate to isovalrealdehyde and this aldehyde was assumed to be converted to isovaleryl-CoA [1,2]. Subsequently, an attempt was made to extract an enzyme catalyzing the latter reaction which might be a CoA-linked aldehyde dehydrogenase (aldehyde : $NADP^+$ oxidoreductase, EC 1.2.1.10). However, such an enzyme could not be isolated and instead an NADP-specific CoA-

* Pressent address: Section of Frost Injury in Plants, Institute of Low Temperature Science, Hokkaido University.

** To whom correspondence should be addressed.

independent aldehyde dehydrogenase (EC 1.2.1.4) was isolated and purified to homogeneity.

Although there are numerous reports on aldehyde dehydrogenases, only a few enzymes have been purified to a homogeneous state and few enzymes have been reported to be NADP-specific.

This report describes the purification, crystallization, and some properties of an NADP-linked aldehyde dehydrogenase from *P. vulgaris*.

Materials and Methods

Materials. $NADP^+$ was obtained from Boehringer Mannheim. Protamine sulfate (from salmon), dithiothreitol and Tris were obtained from Nakarai Chemicals, Ltd. DEAE-cellulose was obtained from Brown. Sephadex G-200 was product of Pharmacia. Standard marker proteins for molecular weight determination were purchased from Mann Research Laboratories, Inc. Isovaleraldehyde and acetaldehyde were obtained from Tokyo Kasei Kogyo Co., Ltd. and Wako Pure Chemicals, respectively, propionaldehyde and benzaldehyde from Nakarai Chemicals, Ltd. All aldehydes were reagent grades and used without further distillation. All other chemicals were used in the purest grade. Hydroxyapatite was prepared according to the method of Tiselius et al. [3].

Enzyme assay. The activity of aldehyde dehydrogenase was determined following the rate of NADPH formation at 340 nm by a Hitachi Perkin-Elmer Type 139 spectrophotometer. The standard reaction mixture consisted of following components in a final volume of 2 ml: 20 mM Tris · HCl buffer, pH 8.7; 15 mM ammonium sulfate; 0.12 mM $NADP^+$; 0.2 mM isovaleraldehyde; and an appropriate amount of enzyme. The reaction was started by addition of substrate at about 30° C, and optical density changes were recorded at 15-s intervals. The unit of activity was defined as the amount of enzyme which reduced 1 μmole of $NADP^+$ per min. Specific activity was defined as units of enzyme activity per mg of protein.

Protein determination. Protein concentration was determined by the method of Lowry et al. [4] with bovine serum albumin as a standard.

Disc gel electrophoresis. Polyacrylamide gel electrophoresis was performed essentially according to the method described by Davis [5]. 5% acrylamide gel was used as a separating gel. The enzyme sample containing 0.5 M sucrose and 10 mM β-mercaptoethanol was directly laid on the spacer gel. 50 mM Tris/glycine buffer, pH 8.3, containing 10 mM β-mercaptoethanol was used as buffer system. The electrophoresis was carried out at room temperature with a current intensity of 1.5 mA per tube for about 100 min.

Molecular weight determination. The molecular weight was determined by gel filtration essentially according to the method of Andrews [6] using Sephadex G-200 (superfine). The proteins used as standard markers were γ-globulin, albumin, ovalbumin, chymotrypsinogen, myoglobin and cytochrome *c*.

Ultracentrifugation. Ultracentrifugal analysis was performed by a Spinco model E analytical ultracentrifuge at 56 100 rev./min and 20° C with Schlieren optics.

Culture of bacteria. P. vulgaris was cultivated in 4 l of the liquid nutrient medium which consisted of 1% each of peptone and broth. After incubation by

a rotary shaker at 37° C for 15 h, the bacterial cells were harvested and washed three times with deionized water by centrifugation. About 8 g wet weight of bacteria were obtained per liter of the medium.

Results

Purification procedure

All purification procedures were performed at 0—4° C unless otherwise specified.

Sonic extract. Aliquots of about 8 g of washed bacterial cells were suspended in 40 ml of 20 mM potassium phosphate buffer, pH 7.0, and disrupted by 20 kc sonic oscillation for 8 min at 0—2° C. Supernatant from similar aliquots obtained by centrifugation at 15 000 × *g* for 30 min were combined.

Protamine sulfate treatment. About 17 ml of 1% protamine sulfate solution was added to 140 ml of the sonic extract with stirring and the precipitate obtained by centrifugation was discarded.

Acetone precipitation. The protamine-treated supernatant was fractionated with cold acetone (—20° C). The fraction precipitating between 33% and 60% of acetone was collected, dissolved in about 100 ml of 20 mM potassium phosphate buffer, pH 7.0. After gently stirring for 30 min, insoluble materials were removed by centrifugation.

Ammonium sulfate fractionation. The acetone fraction (120 ml) was fractionated with powdered ammonium sulfate. The fraction precipitating between 20% and 60% saturation of ammonium sulfate was collected, dissolved in 26 ml of 20 mM potassium phosphate buffer, pH 7.0, and dialyzed overnight against 1 l of 20 mM potassium phosphate buffer, pH 6.1, with several changes of buffer. Insoluble materials were centrifuged off.

Hydroxyapatite column chromatography. The dialyzed enzyme solution was applied to a hydroxyapatite column (2.4 × 12 cm) previously equilibrated with 20 mM potassium phosphate buffer containing 1 mM EDTA and 3 mM dithiothreitol, pH 6.1, and after washing with the same buffer, enzyme was eluted successively with M/10, M/9 and M/8 potassium phosphate buffer containing 1 mM EDTA and 3 mM dithiothreitol, pH 6.1. Aldehyde dehydrogenase activity emerged in M/9 eluted fractions. Active fractions were combined and dialyzed against 20 volumes of 20 mM potassium phosphate buffer containing 1 mM EDTA and 3 mM dithiothreitol, pH 6.1, with stirring for 18 h.

DEAE-cellulose column chromatography. The dialyzed enzyme was loaded onto DEAE-cellulose column (1.4 × 10 cm) equilibrated with 20 mM potassium phosphate buffer containg 1 mM EDTA and 3 mM dithiothreitol, pH 6.1. After washing with the same buffer, elution was performed with 0.12 M KCl in the same buffer. Active fractions were combined and concentrated to about 2 ml using a collodion bag.

Sephadex G-200 gel filtration. The concentrated enzyme was loaded onto the Sephadex G-200 column (2.4 × 36 cm) equilibrated with 20 mM potassium phosphate buffer containing 1 mM EDTA and 3 mM dithiothreitol, pH 6.1, and eluted with the same buffer at a flow rate of 2.5 ml per h. A summary of the purification is shown in Table I. The purified enzyme preparation was stored at 0—4° C in 20 mM potassium phosphate buffer containing 1 mM EDTA

TABLE I

PURIFICATION OF ALDEHYDE DEHYDROGENASE FROM *PROTEUS VULGARIS*

Fraction *	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purity
Sonic extract	140	1148	585.2	0.51	100	1
Protamine sulfate	150	552	557.6	1.01	95.3	2
Acetone ppt.	120	201	365.6	1.82	62.5	3.6
Ammonium sulfate	32	141	347.3	2.46	59.3	4.8
Hydroxyapatite	52	23.7	285.2	12.0	48.7	23.5
DEAE-cellulose	29	18.7	272.0	14.5	46.5	28.4
Sephadex G-200	25	14.0	204.2	14.6	34.9	28.6

* Each fraction was prepared as described in the text.

and 8 mM dithiothreitol, pH 6.1, without significant loss of enzymatic activity for a period of one month.

Crystallization. About 20 ml of the purified enzyme was concentrated to about 1 ml. To a concentrated enzyme solution was added powdered ammonium sulfate to about 20% saturation. Thenceforth, powdered ammonium sulfate was gradually added with great care until cloudiness was visible. Then a little amount of distilled water or 20 mM potassium phosphate buffer, pH 6.1, was added until the cloudiness just disappeared. The enzyme solution was kept at room temperature over a period of one week and large crystals were detected if the crystal-

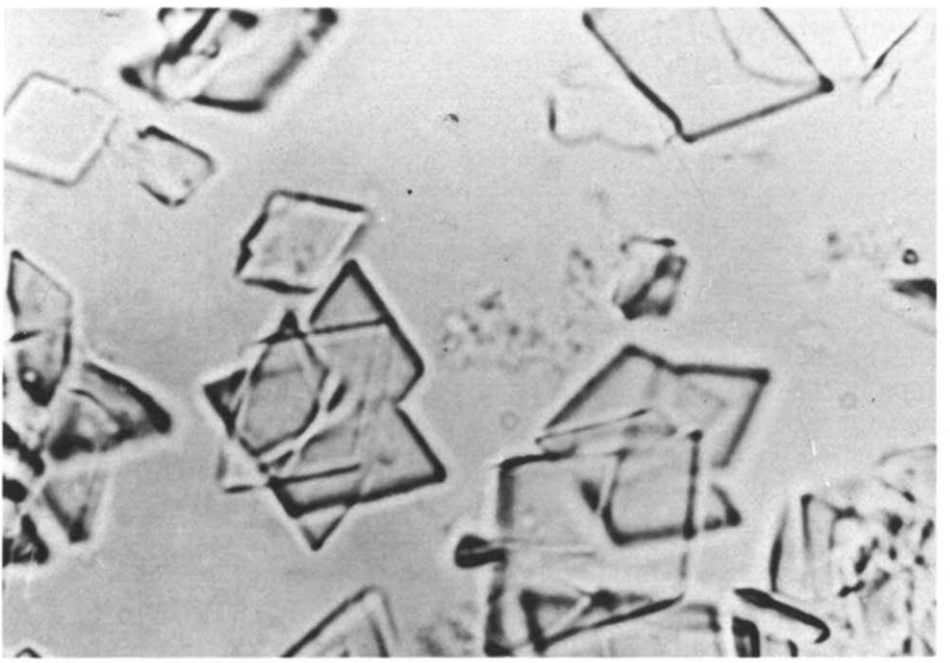

Fig. 1. Photograph of the crystallized aldehyde dehydrogenase at an initial magnification of X 1500.

lization was successful. A typical photograph of the crystallized enzyme is represented in Fig. 1. However, catalytic activity was lost after crystallization.

Enzyme homogeneity. Two different purification steps of the enzyme were examined in respect of their purities by polyacrylamide gel electrophoresis. The electrophoretic pattern of the enzyme after Sephadex G-200 gel filtration showed a single band (Fig. 2). However, a faint band with low mobility appeared occasionally, but was considered to be an artifact, since usually it did not appear in the same enzyme preparation.

Furthermore, an ultracentrifugal analysis of the purified enzyme preparation was performed, and showed only a single mathematical peak at any given time.

Properties of the enzyme

Requirement of monovalent cations. To examine the effect of cations on the enzyme activity, the purified enzyme was used after dialysis for 18 h against 20 mM Tris · HCl buffer, pH 7.0, containing 1 mM dithiothreitol. As shown in Table II, the enzyme reaction completely depended on the presence of mono-

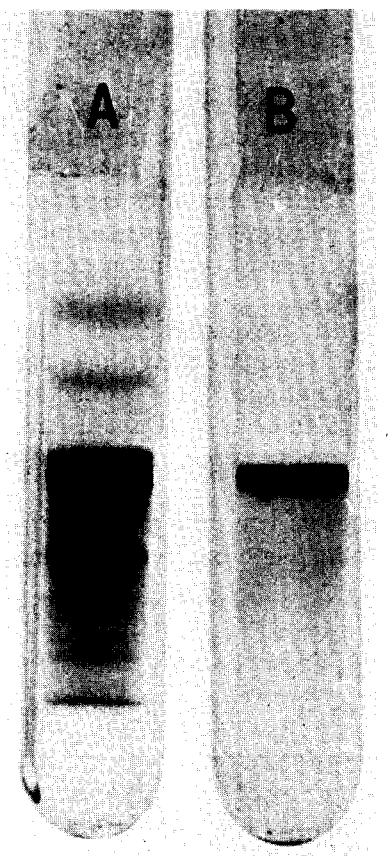

Fig. 2. Polyacrylamide disc gel electrophoresis of aldehyde dehydrogenase. Electrophoresis was carried out as described under Materials and Methods. (A) about 50 μg of the fraction obtained after hydroxyapatite column chromatography; (B) 15 μg of the purified enzyme after Sephadex gel filtration.

TABLE II

MONOVALENT CATION REQUIREMENT FOR ALDEHYDE DEHYDROGENASE ACTIVITY

Assays were as described in Materials and Methods. Final concentration of additions was 3 mM.

Additions	Relative activity (%)
None	0
NH_4Cl	100
RbCl	55
KCl	43
CsCl	12
LiCl	5
NaCl	3

valent cations such as NH_4^+, Rb^+, or K^+, whereas, Cs^+, Li^+ and Na^+ had little or no effect on the enzyme activity. The Michaelis constant (K_m) for NH_4^+ was $3.7 \cdot 10^{-3}$ M, and for Rb^+ and K^+ $1.3 \cdot 10^{-2}$ and $1.6 \cdot 10^{-2}$ M, respectively.

Optimum pH. The enzyme activities were assayed in pH range of 6.4 to 11.5 in a presence of potassium chloride instead of ammonium ion, since the ammonium ion cannot function as cofactor at higher pH. The pH optimum of the enzyme activity was found to be about 9.

Substrate specificity. Although only a few aldehydes were examined in this investigation, as shown in Table III, all aldehydes tested except benzaldehyde served as substrates for this enzyme. Moreover, propionaldehyde and acetaldehyde were better substrates than isovaleraldehyde for the enzyme. As these aldehydes rather inhibited the enzyme activities in higher concentrations, apparent K_m values were determined in the range of lower concentrations of substrates. The apparent K_m values are shown in Table III.

Coenzyme specificity. Dehydrogenations of both isovaleraldehyde and acetaldehyde using the sonic extracts and the purified aldehyde dehydrogenase were examined in the presence of NAD^+ (Sigma Chemical Co.) instead of $NADP^+$. No reaction was found to occur. Similarly, 250 μM CoA (Daiichi Pure Chemicals Co., Ltd.) had not any effect on these hydrogenations in the presence of NAD^+ or $NADP^+$.

Molecular weight. The purified aldehyde dehydrogenase and the marker proteins were dissolved in 1 ml of 20 mM potassium phosphate buffer containing

TABLE III

SUBSTRATE SPECIFICITY OF ALDEHYDE DEHYDROGENASE

Enzyme activities were assayed as described in Materials and Methods. K_m values were calculated from Lineweaver-Burk plots.

Substrate	Concentration (M)	Relative activity (%)	K_m (M)
Propionaldehyde	$1.0 \cdot 10^{-4}$	181	$1.7 \cdot 10^{-5}$
Acetaldehyde	$1.2 \cdot 10^{-3}$	131	$4.0 \cdot 10^{-5}$
Isovaleraldehyde	$2.0 \cdot 10^{-4}$	100	$3.0 \cdot 10^{-5}$
Benzaldehyde	$6.4 \cdot 10^{-5}$	6	—

1 mM dithiothreitol and 0.1 M KCl, pH 6.1, and applied to a Sephadex G-200 column (2.4 × 41 cm) equilibrated with the same buffer. The logarithms of the molecular weights were plotted against the elution volumes of the marker proteins. The molecular weight of the aldehyde dehydrogenase was estimated to be about 130 000.

Discussion

In this study, an NADP-linked aldehyde dehydrogenase (EC 1.2.1.4) was isolated from *Proteus vulgaris*, instead of CoA-linked aldehyde dehydrogenase which was previously presumed to function in this bacteria. The aldehyde dehydrogenase was purified to the stage of homogeneity as judged by ultracentrifugation and gel electrophoresis. The enzyme was also crystallized from ammonium sulfate solution at room temperature, but the crystallized enzyme did not have any catalytic activity as reported by Steinman and Jakoby [7] for yeast aldehyde dehydrogenase (EC 1.2.1.3). Although the crystallization procedures were performed under lower temperature (2—4°C) to avoid enzyme inactivation, the enzyme could never be crystallized under such conditions.

Aldehyde dehydrogenase usually have broad substrate specificities, while highly specific enzymes such as glutaric semialdehyde dehydrogenase [8], malonic semialdehyde dehydrogenase [9] and so on, have been also reported. Our aldehyde dehydrogenase was assayed only with isovaleraldehyde during the progress of purification, but the purified enzyme was found to be more active towards propionaldehyde and acetaldehyde than isovaleraldehyde. Considering K_m values for these aldehydes, it is doubtful that the enzyme in living cells is operating exclusively for the branched chain amino acid degradation. Although further metabolism of free isovaleric acid is to be studied, it may be assumed that there are different degradative pathways of L-leucine in *Proteus* from the established pathway.

As for NADP-linked aldehyde dehydrogenase, only *Acetobacter* [10] and yeast [11] enzymes have been reported as far as we know. These enzymes were only partially purified and activated by addition of bivalent cations such as Ca^{2+}, Mg^{2+}, Mn^{2+} and so on, while this enzyme was activated by monovalent cations such as NH_4^+, K^+ and Rb^+. These *Acetobacter* and yeast enzymes may function in ethanol metabolism, since acetaldehyde is oxidized most rapidly by these enzymes. As to this enzyme, proprionaldehyde is the best substrate and isovaleraldehyde is oxidized at the considerable high rate, so a physiological role of this enzyme seemed to be complex.

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