PERILLYL ALDEHYDE DEHYDROGENASE FROM A SOIL PSEUDOMONAD*

N. R. Ballal, P. K. Bhattacharyya,& P. N. Rangachari

National Chemical Laboratory, Poona-8, India.

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A soil pseudomonad (PL-strain) capable of utilising limonene, α-pinene or β-pinene, Δ^1 p-menthene or p-cymene as its sole source of carbon was isolated by enrichment culture technique. Many neutral and acidic products were isolated from the limonene fermentation broth (1). The major pathway (Figure 1) for limonene catabolism which would provide the necessary substrates for the amphibolic enzymes has been shown to be by a progressive oxidation of the 7-methyl group to the carboxyl through the corresponding alcohol and aldehyde (2). The degradation pathway from α and β-pinenes presumably involves Δ^1 p-menthene as an intermediate, the 7-methyl group of which is also progressively oxidised by a parallel sequence. The methyl group of the aromatic hydrocarbon p-cymene undergoes oxidation to the carboxyl group.

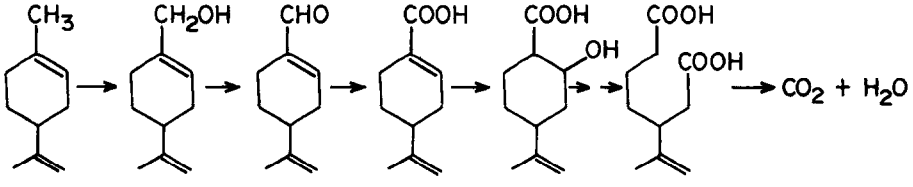

Fig. 1

All the enzymes of the limonene catabolism sequence upto β-isopropenyl pimelic acid have been qualitatively demonstrated in the cell-free sonicates of the bacterium (2). The alcohol dehydrogenase which exhibits certain features in its substrate requirements has been partially purified and its properties studied (3) and is

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capable of oxidising the 7-hydroxymethyl group of dihydro perillyl alcohol and cumic alcohol but inactive towards the ordinary alcohols. It was therefore, considered necessary to study the aldehyde dehydrogenase of this sequence particularly with respect to the substrate specificity. In the present communication the partial purification and some of the properties of the aldehyde dehydrogenase which appears to be different from the other aldehyde dehydrogenases (4,5) are reported.

The organism was grown in a mineral salts medium as described by Dhavalikar & Bhattacharyya (1). The harvested and washed cells stored at -25°C were suspended (1 g of wet cells to 10 ml buffer) in phosphate buffer (0.05 M, pH 7.0) containing cysteine (0.005 M). The sonication was carried out for 15 min in a Raytheon sonic oscillator (10 Kc). Unbroken cells and cell debris were removed by centrifugation at 10,000 g for 30 min. The aldehyde dehydrogenase activity in the supernatant was followed spectrophotometrically by measuring the amount of NADH formed. The reaction mixture contained in 1 ml.: Tris-chloride (pH 8.6) 50 μmoles, NAD 0.5 μmole, cysteine 10 μmoles, enzyme sufficient to give an optical density change of 0.04 to 0.05 per 30 seconds. The above components were incubated for 5 min. at room temperature (28°C) and perillaldehyde, 2.0 μmoles, were added to start the reaction. The amounts of NAD and of the aldehyde used were found sufficient to saturate the enzyme under the conditions of assay, and under these conditions the activity followed a zero order kinetics. A unit of enzyme is defined as the number of μ moles of NADH formed per min. under the assay conditions and specific activity is the activity per mg. protein. Protein was estimated according to the procedure of Lowry et al. (6) with crystalline bovine serum albumin (Sigma) as the working standard and the color was read at 500 mμ in a Beckman DU spectrophotometer.

The cell-free extract (specific activity 0.25–0.35) was carried

through the following steps to give a 10-fold purification of the aldehyde dehydrogenase: (i) treatment with streptomycin sulfate (1%) at pH 6.0 to remove nucleic acid (ii) first fractionation with solid ammonium sulfate (iii) adsorption and elution from a hydroxyl apatite gel column (gel prepared according to Tiselius et al. (7)) and (iv) a second ammonium sulfate fractionation. All operations were carried out at 0° to 4°C and throughout the purification procedure, cysteine (final concentration 0.005 M) was added to the solutions to stabilise the enzyme. The following table gives a typical purification procedure with the specific activities and the recovery of the enzyme.

Steps	Volume (ml.)	Total activity (units)	Total protein (mg.)	Specific activity	Recovery (%)
Sonicate supernatant	65	260	845	0.3	100
Streptomycin sulfate supernatant	210	245	806	0.3	94
1st ammonium sulfate fraction (0.35-0.70)	10	200	325	0.6	77
Hydroxylapatite gel eluate	50	160	64	2.5	62
2nd ammonium sulfate fraction(0.4 - 0.7)	5	150	50	3.0	57

The enzyme preparation with a specific activity of 3.0 was used to study some of the properties. This preparation was not very stable and lost about 25 to 30% of activity at -25°C in 5 days. The loss in activity was much greater if cysteine was omitted from the buffer.

The optimum pH for maximum activity of the aldehyde dehydrogenase was determined to be 10.0 (glycine-sodium hydroxide buffer). At this pH, the Michaelis constant for the substrate was found to be 1.1×10^{-4} M and that for NAD was 2×10^{-4} M. The enzyme was mostly specific for NAD as cofactor while it showed relatively smaller activities with NADP

(6% of that with NAD), with 3-acetylpyridine analog of NAD (12%) and with desamino-NAD analog (13%). It had no activity with 3-pyridine aldehyde analog of NAD and with thionicotinamide analog. Except Hg^{++}, Ag^{+} and Cu^{++}, no other metal ions tested (Mn^{++}, Zn^{++}, Ni^{++}, and Mg^{++}) showed any inhibition of the activity but this inhibition with Hg^{++}, Ag^{+} and Cu^{++} could be prevented by preincubation of the enzyme with cysteine (0.01 M). Metal complexing agents like EDTA, orthophenanthroline, $\alpha - \alpha'$ dipyridyl and pyrophosphate had no effect on the dehydrogenase activity even at relatively high concentrations of the order of 2×10^{-3} M to 2×10^{-2} M. Exhaustive dialysis of the enzyme in the presence of cysteine did not bring about loss in activity of the enzyme. The above properties seem to indicate the absence of any metal requirement for this enzyme as compared to the two dehydrogenases isolated from yeast. One of them is activated by potassium or rubidium (8) and the other by magnesium (9). The enzyme was dialysed to remove cysteine and the effect of sulfhydryl reagents on such a preparation was carried out. p-Hydroxymercury benzoate inhibited completely the enzyme activity at as low a concentration as 2×10^{-5} M but the inhibition could be prevented by prior incorporation of cysteine (0.01 M) in the system. Iodoacetate inhibited only at high concentrations viz. 2×10^{-2} M (60%) and this inhibition could also be similarly prevented by incorporation of cysteine. Arsenite did not have any effect even at concentrations of the order of 10^{-2} M and in this respect this enzyme seems to differ from other aldehyde dehydrogenases reported in literature (4, 10). The enzyme shows activity in the absence of cysteine but maximum activity is obtained only by preincubation with cysteine, glutathione or 2-mercapto ethanol (10^{-2} M). Coenzyme A has no effect on activation of a dialysed enzyme at the concentrations studied (10^{-3} M). The requirement of a mercaptan for activity, the inactivation of the enzyme by low concentrations of sulfhydryl reagents and the prevention of this inactivation by incor-

poration of cysteine in the assay system indicate clearly the essentiality of -SH groups for the dehydrogenase reaction. Besides perillaldehyde, the enzyme was found to be active on a number of other aldehydes like phellandral, cumic aldehyde, benzaldehyde, m- and p-methyl benzaldehydes and acetaldehyde. The enzyme showed very little activity with o-methyl benzaldehyde. In its substrate specificity, this dehydrogenase seems to resemble the other known aldehyde dehydrogenases like the one from yeast (8, 9), from Pseudomonas fluorescens (4), from rabbit liver (11) and from rat kidney (12). It is noteworthy that no specific structural requirements could be noticed with respect to the substrate specificity of this dehydrogenase as compared to that of the alcohol dehydrogenase from the same organism (3). The product of dehydrogenation with perillaldehyde has been established as perillic acid (2). The dehydrogenase activity test was carried out in the presence of CoA and a hydroxamate test (13) on the reaction product was found to be negative. Treatment of the enzyme with Dowex-1 (Cl form) did not impair the activity. These tests were also carried out with the crude cell-free extracts with the same results. These clearly show the non-participation of CoA in the dehydrogenase reaction. Since the product of the dehydrogenase activity had been shown to be the free acid, reversibility of the reaction was not attempted.

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