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AROMATIC ALCOHOL DEHYDROGENASE FROM POTATO TUBERS

D. D. DAVIES, E. N. UGOCHUKWU, K. D. PATIL and G. H. N. TOWERS

School of Biological Sciences, University of East Anglia, Norwich NOR 88C

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Key Word Index—*Solanum tuberosum*; (Solanaceae); potato; aromatic alcohol dehydrogenase; alcohol dehydrogenase; Hammett equation.

Abstract—The purification of NADP specific aromatic alcohol dehydrogenase is reported. The properties of the enzyme suggest that it should be classified as E.C. 1.1.1.2. Kinetic constants for a number of substrates are reported. The relative rates of reaction of a variety of substituted benzaldehydes have been found to correlate with Hammett's sigma values yielding a biphasic relationship. The physiological significance of the enzyme is briefly discussed.

INTRODUCTION

ALIPHATIC alcohol dehydrogenases have been shown to oxidize a wide range of alcohols.[1] Alcohol dehydrogenases isolated from liver,[2] yeast[3] and potatoes[4] have been shown to be active with aromatic alcohols. However, little work on aromatic alcohol dehydrogenases has been published.

A benzyl alcohol dehydrogenase has been isolated from *Mycobacterium tuberculosis*[5] which is active with NAD and NADP whilst an NAD specific benzyl alcohol dehydrogenase has been purified from *Pseudomonas putida*.[6,7] An enzyme considered to be perillyl alcohol dehydrogenase has been isolated from a soil pseudomonad[8] and found to be active with a number of aromatic alcohols.

An NADP specific aromatic alcohol dehydrogenase has been purified from *Neuospora crassa*[9] and an NADP specific *m*-hydroxybenzyl alcohol dehydrogenase has been purified from *Penicillium urticae*.[10] An enzyme which transfers hydrogen from NADPH to aromatic aldehydes and ketones has been purified from rabbit kidney cortex.[11] However, for reasons which are not apparent this enzyme does not catalyse the oxidation of aromatic alcohols.

The present paper is concerned with the purification and properties of an NADP specific aromatic alcohol dehydrogenase from potato tubers (*Solanum tuberosum*).

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RESULTS

Partial Purification of Potato Aromatic Dehydrogenase

The results of the procedure given in the experimental section are presented in Table 1. The crude extract contains at least three alcohol dehydrogenases and the data of Table 2 shows that purification has yielded a preparation of aromatic alcohol dehydrogenase relatively free from activity with aliphatic substrates.

TABLE 1. PURIFICATION OF AROMATIC ALCOHOL DEHYDROGENASE FROM POTATO TUBERS*

Extract fraction	Volume (ml)	Protein (mg/ml)	Enzyme activity (units/ml)	Specific† activity	Purification
Extract	750	3·6	0·15	0·041	1·0
$(NH_4)_2SO_4$ (0–35%)	50	22·6	0·175	0·008	0·2
$(NH_4)_2SO_4$ (35–50%)	50	15·3	0·35	0·023	0·5
$(NH_4)_2SO_4$ (50–60%)	50	4·3	0·27	0·063	1·5
50–60% dialysed	70	2·3	0·21	0·09	2·2
DEAE peak	4	0·02	0·03	1·5	36

* Activity was measured with benzaldehyde and NADPH as substrates.
† For experimental details see text.

The separation of activities obtained by ion exchange chromatography on a column of DE52 cellulose is shown in Fig. 1. Three peaks of activity are apparent; peak 1 represents an aliphatic alcohol dehydrogenase possessing minor activity towards aromatic substrates; peak 2 represents a NADP specific terpene alcohol dehydrogenase; peak 3 represents the NADP specific aromatic alcohol dehydrogenase which is the subject of this communication.

TABLE 2. ACTIVITY OF ALIPHATIC AND AROMATIC ALDEHYDES WITH TWO OF THE ALCOHOL DEHYDROGENASES PRESENT IN POTATO TUBERS*

Aldehyde	Activity with NADH and Peak 1 (ΔE_{340}/min/ml)	Peak 3 (ΔE_{340}/min/ml)	Ratio Peak 3/Peak 1
Benzaldehyde	1·4	10·5	7·5
Cinnamaldehyde	2·5	7	2·8
3-Phenylpropionaldehyde	10	4	0·4
Acetaldehyde	125	1·5	0·01

* For experimental details see text.

The specificities of the purified fractions (peaks 1 and 3) towards aliphatic and aromatic substrates are not absolute (Table 2). However, if we consider that in the sequence benzaldehyde, cinnamaldehyde, 3-phenylpropionaldehyde, acetaldehyde, the aldehydes are decreasingly aromatic in character, then the relative activities of the four aldehydes with peak 3 is in the same sequence, i.e. decreasing activity with decreasing aromaticity. Peak 3 is thus considered to represent an NADP specific aromatic alcohol dehydrogenase. Peak 1 on the other hand shows activity with the four aldehydes in the reverse order and is thus considered

to be an NAD specific aliphatic alcohol dehydrogenase.[4] Peak 2 shows activity with citral, and geraniol and is considered to be a terpene alcohol dehydrogenase. Cyclohex-4-ene carboxaldehyde also acts as a substrate for the terpene alcohol dehydrogenase but appears to have little or no activity with the aromatic alcohol dehydrogenase.

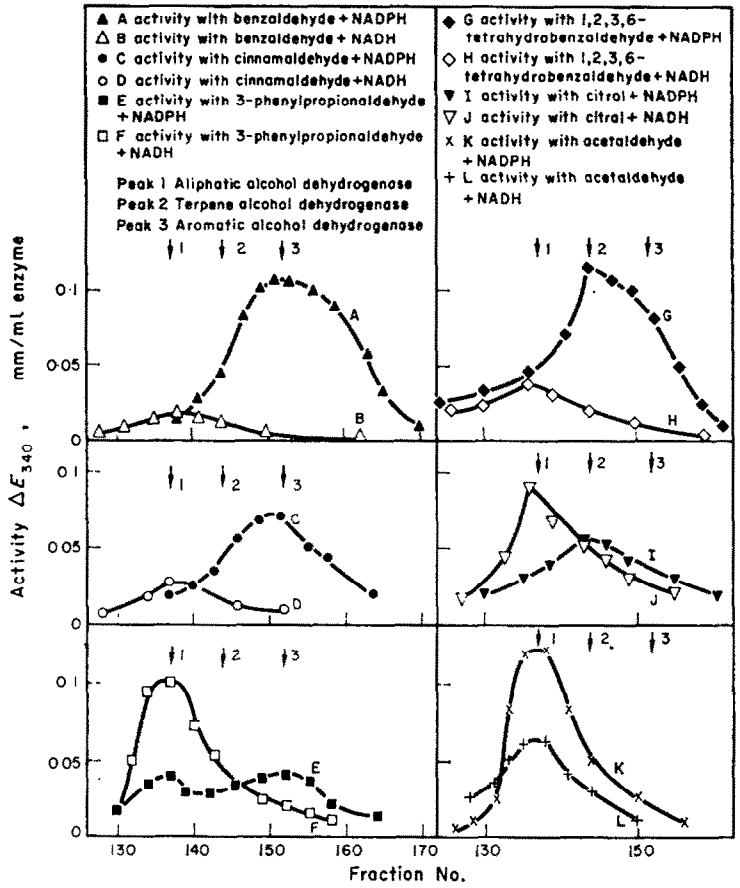

FIG. 1. SEPARATION OF ALCOHOL DEHYDROGENASES FROM POTATO TUBERS BY ION EXCHANGE CHROMATOGRAPHY.
For experimental details see text. The scale for activity with acetaldehyde + NADH is 10× all other scales.

Stability

Preparations were stabilised by including mercaptoethanol (5 mM) in all buffers and by dissolving sucrose (2·5 g/10 ml) in the final preparation. Such preparation lost little activity over a period of 2 weeks.

Kinetic Constants

The standard buffer used was Tris-HCl. However, Tris is a trihydric alcohol and thus a potential substrate. Mahler[12] has reported that Tris is a substrate for liver alcohol dehydrogenase and competes with ethanol for active sites on the enzyme. Further, Tris reacts with

[12] H. R. MAHLER, *Ann. N.Y. Acad. Sci.* **92**, 426 (1961); **126**, 933 (1961).

aldehydes[13] and its use with substrates containing a reactive carbonyl group should be questioned. Consequently we have examined the effect of Tris on the potato aromatic alcohol dehydrogenase. Tris does not serve as a substrate nor does it inhibit the enzyme. Incubation of Tris with an aldehyde and substrate might be expected to lead to transformation of the aldehyde and to a reduction in the rate of reaction when enzyme is subsequently added. Experiments to test this showed that incubation of Tris with benzaldehyde for periods up to 30 min does not lead to a reduction in the rate of NADPH oxidation when the enzyme was subsequently added. In fact incubation with Tris produced a slight but significant increase in the rate of reaction. Some kinetic constants determined in the presence of Tris are given in Table 3.

TABLE 3. KINETIC CONSTANTS OF POTATO AROMATIC ALCOHOL DEHYDROGENASE WITH VARIOUS SUBSTRATES

Variable substrate	Fixed substrate	Conc. of fixed substrate	K_m (apparent) (mM)	V_{max} as % of activity with benzaldehyde
Benzaldehyde	NADPH	0·133 mg/m	0·34	100
Cinnamaldehyde	NADPH	0·133 mg/ml	0·22	266
3-Phenylpropionaldehyde	NADPH	0·133 mg/ml	0·55	133
Benzyl alcohol	NADP	0·133 mg/ml	4·35	33
NADP	Benzyl alcohol	2 mM	0·18	33
NADPH	Benzaldehyde	2 mM	0·017	66

Conditions of assay; for aldehydes Tris buffer pH 7·4, 0·05 M; for benzyl alcohol Tris buffer pH 8·4 0·05 M. NADP or NADPH (0·133 mg/ml) and enzyme 0·2 ml in a final vol. of 3 ml. Temp 30°.

The plots on which Table 3 is based were in all cases linear.

Effect of Ring Substitution on the Rate of Reaction

Many of the aromatic aldehydes and alcohols which occur in plants are ring substituted and therefore we have examined a number of such compounds. The kinetic constants for various substituted benzaldehydes were determined. Because of the relatively insoluble

TABLE 4. KINETIC CONSTANTS OF POTATO AROMATIC ALCOHOL DEHYDROGENASE WITH VARIOUS SUBSTITUTED BENZALDEHYDES

Benzaldehyde	K_m (apparent) (mM)	V_{max} as % of activity with benzaldehyde	Benzaldehyde	K_m (apparent) (mM)	V_{max} as % of activity with benzaldehyde
p-Methyl-	0·30	100	*o*-Bromo-	0·25	166
m-Hydroxy-	1·10	133	*m*-Bromo-	0·16	166
p-Hydroxy-	0·09	100	*p*-Bromo-	0·22	100
o-Chloro-	0·20	100	*o*-Fluoro-	0·27	100
m-Chloro-	0·10	166	*m*-Fluoro-	0·42	200
p-Chloro-	0·34	233	*p*-Fluroro-	0·26	100
			o-Nitro-	0·05	166
			m-Nitro-	0·21	166
			p-Nitro-	0·04	1066

Conditions of assay as in text.

[13] P. F. DUGGAN, D. M. X. DONNELLY and D. P. MELODY, *Irish J. Med. Sci.* **460**, 163 (1964).

nature of the substituted aldehydes, they were brought into solution using methanol (final concentration of methanol 0·33–1·6%). At these concentrations methanol produced negligible inhibition. Assays were carried out in Tris buffer (pH 7·4, 0·05 M) with NADPH 0·4 mg in a final vol. of 3 ml. Lineweaver–Burk plots were linear and the kinetic values are presented in Table 4.

The effect of ring substitituon on the rate of reaction has been examined by Hammett-type plots.[14] In Fig. 2(a) we have plotted log V_{max} against σ, and in Fig. 2(b) log V_{max}/K_m against σ. Plots including K_m values are presented because although the Hammett relationship defines the rate of the catalytic step, the overall rate depends on the binding of substrate to the enzyme and this is reflected in the K_m values. Although the interpretation of these plots is difficult the results are in general agreement with the findings of Blomquist[15] and Culp and McMahon[11] that *p*-substitution with electron-withdrawing groups greatly enhances the rate, while electron donating groups reduce the rate relative to benzaldehyde.

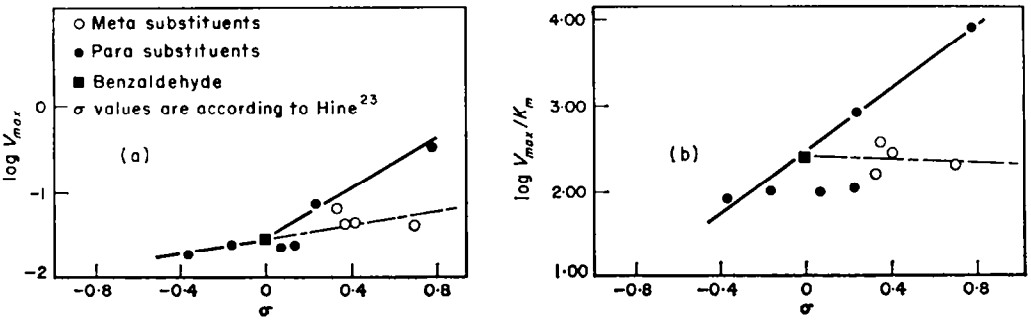

FIG. 2. HAMMETT PLOTS FOR SUBSTITUTED BENZALDEHYDES.

DISCUSSION

Near Linear relationships were obtained in the Hammett-type plots for para substituted benzaldehydes and also for *meta* substituted benzaldehydes [Figs. 2(a) and (b)]. Biphasic plot which is seen in Fig. 2a, may according to Blomquist[15] be explained by a change in mechanism or in the rate determining step of the overall enzyme reaction. Colinearity between various *para* and *meta* substituted benzaldehydes was not observed. We suggest that this is due to steric effects involved in the binding of the substrates to the enzyme. The deviations from strict linearity even within the same substituent position are explicable in terms of differences in binding on the enzyme surface due to differing lipophilicity[15] and perhaps also to conjugative and other effects (e.g. hydroxyl groups would tend to stabilize the aldehyde substrate in preference to the intermediate transition state.[16] There are considerable difficulties in interpreting the results but it is nevertheless possible to make the broad generalization that polar effects are important in determining the overall rate of reaction.

The properties of the enzyme described in this paper suggest that it is an NADP specific aromatic alcohol dehydrogenase and should be classified as E.C. 1.1.1.2. The preparation is active with a wide range of aromatic compounds and the recognition of the physiological

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role of the enzyme depends upon the identification of its physiological substrate. Benzyl alcohol is widely distributed in plants usually as an ester, *o*-hydroxybenzyl alcohol has a restricted distribution, being best known as the aromatic component of the glycoside salicin. Similarly benzaldehyde is best known as the aromatic component of the cyanogenetic glycoside amygdalin. The aromatic alcohol dehydrogenase may play a role in the interconversion of benzaldehyde and benzyl alcohol and in the interconversion of various substituted forms. However, a more likely role for the enzyme lies in the metabolic route from cinnamic acid to lignin. Cinnamic acid formed from phenylalanine by phenylalanine ammonia lyase[17] is the precursor of *p*-hydroxycinnamic acid, ferulic acid and sinapic acid, which in feeding experiments are efficient precursors of lignin.[18] The postulated route involves the reduction of the aromatic acids via the aldehydes to the corresponding alcohols, and subsequent incorporation into lignin.

The enzyme or enzymes catalysing the reduction of the three acids to their corresponding aldehydes has not been demonstrated in plants. However, Gross and Zenk[19] have isolated an aromatic aldehyde dehydrogenase from *Neurospora* which in the presence of ATP and NADPH converts benzoic acid to benzaldehyde. If a similar enzyme is present in plants the alcohol dehydrogenase reported here could function as the last enzyme in the metabolic sequence leading to the synthesis of the proposed precursors of lignin.

Since the reduction of aromatic aldehydes to aromatic alcohols catalysed by aromatic aldehyde reductase of rabbit is irreversible, the direction of the reaction is the same as proposed for the plant enzyme. However, the benzyl alcohol dehydrogenase isolated from *Pseudomonas putida*[7] is thought to function in the reverse direction. There may well be a number of aromatic alcohol dehydrogenases and the situation may parallel yeast aliphatic alcohol dehydrogenase where one enzyme functions in the production of ethanol the other in ethanol utilization.[20,21] These considerations remain problems for the future.

EXPERIMENTAL

Materials. NAD, NADH, NADP and NADPH were obtained from Boehringer Corp. (London) Ltd., London W5. Aromatic aldehydes were obtained from British Drug Houses, Poole, Dorset, U.K. and redistilled under N_2 immediately prior to use. Cyclohex-4-ene carboxaldehyde and 3-phenylpropionaldehyde were gifts from Dr. D. Land, Food Research Institute, Colney Lane, Norwich, Norfolk, U.K. Other chemicals were of the best reagent grade obtainable.

Enzyme purification. An extract of potato tubers was fractionated with $(NH_4)_2SO_0$ and purified by ion exchange chromatography on DE 52 as previously described.[4] The purification achieved is shown in Table 1.

Enzyme assays. The standard assay was carried out at pH 7·4 by measuring the decrease in extinction at 340 nm associated with the oxidation of NADP. The cuvette (light path 1 cm) contained Tris-HCl buffer (pH 7·4, 0·05 M) NADPH (0·4 mg) and enzyme (0·1 ml) in a vol. of 2·9 ml. The reaction was started by the addition of 0·1 ml of a saturated solution of benzaldehyde. Under these conditions the relation between reaction rate and enzyme concentration was linear. Assays were carried out at 30° using a Unicam SP 500 spectrophotometer. To compare the activity of enzyme fractions with various aldehydes, benzaldehyde was replaced with cinnamaldehyde (3×10^{-2} M) or 3-phenylpropionaldehyde (10^{-2} M) or cyclohex-4-ene carboxaldehyde (3×10^{-2} M) or citral (sat. soln) or acetaldehyde (3×10^{-2} M). A unit of enzyme activity is defined as the amount of enzyme oxidising 1 μmol of NADPH/min—this corresponds to a change in E_{340} of 2·07/min. Specific activity is defined as the number of enzyme units per mg of protein. Protein was determined by the method of Warburg and Christian.[22]

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