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PROPERTIES OF THREE SETS OF ISOENZYMES OF ALCOHOL DEHYDROGENASE ISOLATED FROM BARLEY (*HORDEUM VULGARE*)

ROBERT G. MAYNE* and PETER J. LEA

Department of Biochemistry, Rothamsted Experimental Station, Harpenden, Herts AL5 2JQ, U.K.

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; alcohol dehydrogenase; properties; three isoenzymes.

Abstract—Three sets of isoenzymes of alcohol dehydrogenase were separated from root and shoot tissue of *Hordeum vulgare* by DEAE-cellulose chromatography. Set I showed only one band of ADH activity after polyacrylamide gel electrophoresis; Set II—two and Set III—three, making a total of six discernable bands. Only one set (I) was detected in the dry seed and one set (III) in the M9 (*Adh-1-null*) mutant available in tissue culture. The sets were found to have identical molecular weights (90 000), were all located in the cytoplasm but showed small differences in pH optima and substrate specificity. The affinity for ethanol (K_m value, mM) varied between Set I (27.5), Set II (7.2) and Set III (3.5), whilst the affinity for NADH varied five-fold between the three sets. A dimeric quaternary structure was inferred from the random reassociation of enzyme subunits after dissociation in high ionic strength buffer.

INTRODUCTION

Ethanol is a major fermentation product in plants subjected to flooding [1]; the second step in the conversion of pyruvate to ethanol is catalysed by alcohol dehydrogenase (ADH; EC 1.1.1.1.) which reduces acetaldehyde and regenerates NAD. The role of ADH in flood-tolerant and flood-intolerant plants has been extensively discussed [3, 4]. During periods of anoxia the level of ADH activity in many plants [2, 3], including barley [5], increases. Higher plants often contain more than one form of ADH and isoenzymes have been reported in a number of species [6–9]. The most detailed studies to date have been carried out with maize isoenzymes, which have been used to examine the regulation of gene expression in higher plants [10–12]. A two-gene model has been proposed whereby the products of two unlinked genes randomly dimerise to form three "sets" of ADH isoenzymes—two homodimers and a heterodimer [10]. Harberd and Edwards [13, 14], on the basis of mutational analysis of the barley ADH isoenzyme system, proposed an analogous two-gene model for barley. Brown *et al.* [15, 16] found two *Adh* loci in *Hordeum spontaneum* which were tightly linked in contrast to the unlinked maize genes. The M9 mutant of barley was diagnosed as *Adh-1-null* by electrophoretic analysis [14, 17]. Mutant seeds were found to be more resistant than wild type seeds to allyl alcohol, which is converted by the action of ADH to the highly toxic acrylaldehyde. The increase in ADH activity in the root during anaerobiosis was much less marked in the mutant than in the wild type seedlings.

The aim of this work was to separate the isoenzymes of ADH present in anaerobic barley plants and to examine their properties and subunit structure.

RESULTS

Separation of isoenzymes of ADH

Three peaks of ADH activity were separated from flooded barley root tissue after chromatography on DEAE-cellulose, distinct shoulders being obvious on the second and third peaks (Fig. 1, top). The separation corresponded directly to the separation by polyacrylamide gel electrophoresis (PAGE) (Fig. 1, bottom) and the three peaks were denoted as Set I, II and III as depicted. No additional activity was found to elute at other parts of the gradient or in the "wash" fractions. Extracts of dry seeds yielded a single peak of ADH activity in the Set I position after DEAE-cellulose chromatography with a corresponding single band after PAGE. In contrast ADH isolated from M9 cell suspension cultures chromatographed as a peak in the Set III position with three corresponding bands being visible after PAGE. There was no evidence of Set II activity in either the dry seed or M9 extracts.

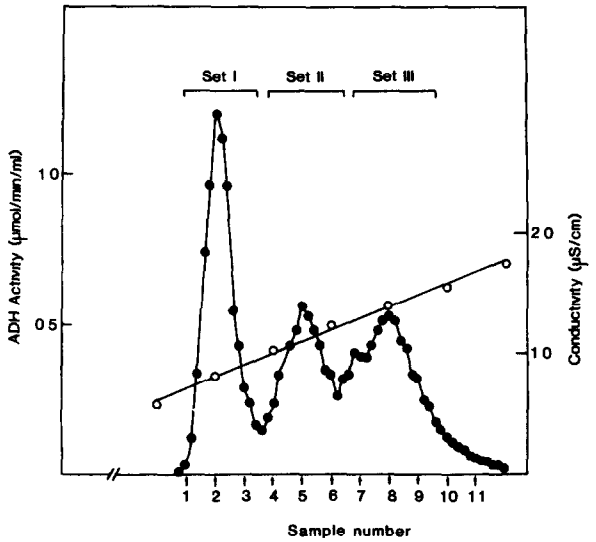
Stability

The addition of glycerol and either DTT or 2-mercaptoethanol to crude extracts of barley root tissue allowed complete recovery of ADH activity after storage on ice or at −20° for 24 hr. Neither ethanol nor acetaldehyde had any significant protective effect. All buffers therefore routinely included 0.1% mercaptoethanol and 10% (v/v) glycerol, which protected all three isoenzymes equally. No changes in the banding pattern after PAGE were detected if the pH of the extracting buffer was varied between 6.8 and 9.1, if phenylmethylsulphonyl fluoride was added, or if the extracts were stored for 24 hr on ice or at −20° prior to electrophoresis. No enhancement of ADH activity was observed during storage of crude extracts.

pH optima

The activity of the three sets of isoenzymes responded in a similar manner to changes in pH (Fig. 2). The major

*Present address: MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, U.S.A.



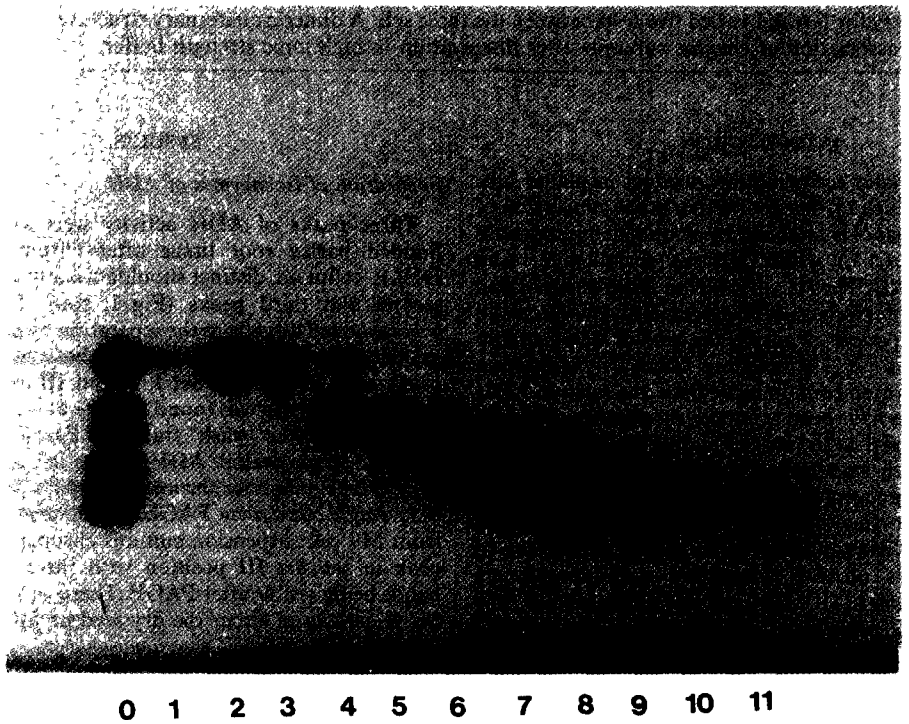


Fig. 1. (Top) Separation of ADH isoenzymes by DEAE-cellulose chromatography. Activity was extracted from the roots of seedlings that had been submerged for 24 hr and then allowed to recover in air for 24 hr. Samples were taken at the point arrowed for electrophoretic analysis. ●—●, ADH activity; ○—○, conductivity of the eluent diluted × 10. (Bottom) Correspondence between separation of ADH isoenzymes by DEAE-cellulose chromatography and PAGE. Samples were subjected to PAGE in a 5% acrylamide gel and stained for ADH activity. O, original extract; other tracks from left to right correspond to samples taken at points arrowed.

difference was the sharp optimum in the forward (ethanol formation) direction of 8.6–8.8 of Set I compared to the broad optima of Sets II and III. Set II appeared to reach a plateau of activity in the reverse direction above pH 9.0. Forward reaction rates were significantly higher than in the reverse reaction and only slight variations in these relative velocities were noted between the three sets. Activity in borate buffer was negligible in both directions.

Substrate specificity

The ability of the isoenzymes of barley root ADH to

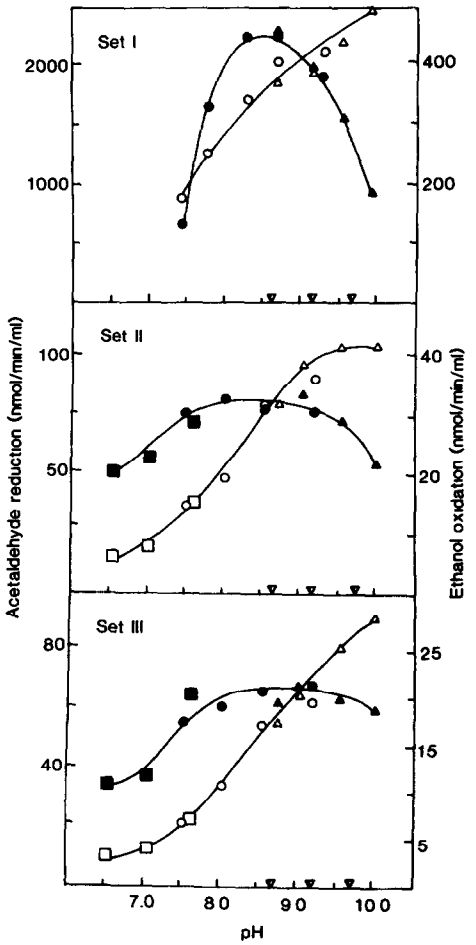


Fig. 2. pH optima of the three ADH isoenzymes of barley. Activity was measured in both the acetaldehyde reduction (closed symbols) and the ethanol oxidation (open symbols) directions. The buffers contained 50 mM KH_2PO_4 (□), 50 mM Tris–HCl (○), 50 mM glycine–KOH (Δ), or 50 mM sodium tetraborate (▽).

Table 1. Substrate specifity of the three isoenzymes of barley roots alcohol dehydrogenase

Substrate	Set I	Set II	Set III
Acetaldehyde	100	100	100
Propionaldehyde[1,2]	58	44	48
Butyraldehyde[1,2]	50	52	72
Nonaldehyde[1,2]	6.3	20	25
Citronellal[1,2]	18	8	14
Cinnemaldehyde[1,2]	0	8	13
Glyceraldehyde	0	12	10
Ethanol	100	100	100
Methanol	0	0	0
n-Propanol	58	85	170
Propan-2-ol	2	2	0
Butan-1-ol[2]	33	54	21
Butan-2-ol[2]	0	0	2
Tertiary butanol	2	8	11
Amylalcohol[2]	35	46	29
Nonanol[2]	0	2	15
Citronellol[1,2]	0	8	14
Cinnamyl alcohol[1,2]	4	8	18
Glycerol	0	0	0
Ethylene glycol	0	0	0

Samples of each set of isoenzyme were prepared by DEAE-cellulose chromatography as shown in Fig. 1. Rates are expressed in relation to those obtained with either ethanol or acetaldehyde as a substrate (= 100). Substrates were added in saturating concentrations, except those marked[1] which were limited by theirvsolubility. Compounds marked[2] absorbed strongly at 340 nm, the wavelength used for the assay.

utilise various alcohols and aldehydes as substrates is shown in Table 1. Set I was unable to utilise a number of compounds as substrate in both directions. Set II had the greatest ability to use butan-1-ol and amylalcohol as substrates. Set III had a marked capacity to use *n*-propanol as a substrate. The figures presented may not reflect the true reaction rates, since many of the substrates and products absorb strongly at 340 nm, and the absence of activity with some of the higher alcohols may only reflect their low buffer-solubility. Formaldehyde was found to oxidise NADH at a very fast rate in the absence of enzyme and was not further studied. The rate of reaction of all three isoenzymes using NADPH was < 3% of that obtained in the presence of NADH.

Kinetic parameters

The K_m values for the substrates ethanol and acetaldehyde and for the cofactors NAD and NADH were obtained for each isoenzyme by the direct linear plot of Eisenthal and Cornish-Bowden [18, 19] using a microcomputer (Table 2). The data were linear if plotted in the form of a double reciprocal plot [20].

Molecular weight

Sedimentation coefficient: All three sets of isoenzyme activity were found to sediment to the same position after sucrose density gradient centrifugation of samples separated on DEAE-cellulose. A mixture of equal activities of the three sets sedimented as a single peak and PAGE analysis showed that all three sets of isoenzymes were present in the peak. The sedimentation coefficient ($S_{20\,w}$) was estimated graphically and found to be in the range of 4.7–4.8.

Stokes radius

A mixture of equal activities of Set I, II and III isoenzymes was chromatographed on Sephacryl S-300, and found to elute as a single peak; the recovery was 98% and the peak fraction contained all forms of the enzyme as detected by PAGE. The Stokes radius, determined graphically, was found to be 4.6 nm.

Assuming a partial specific volume of 0.725 cm^3/g [21] the M_r of barley ADH was calculated as being 89–91 000 and the frictional ratio as 1.55.

Table 2. The Michaelis constants of the isoenzymes of barley alcohol dehydrogenase

Substrate	Set I (Leaves)	Set II (Leaves)	Set III (Leaves)	Set I (Seed)	Set III (M9 Cultures)
Ethanol (mM)	27.5	7.2	3.5	34	4.1
Acetaldehyde (mM)	1.45	1.25	1.7	1.5	1.6
NAD (μM)	73	30	31	70	31
NADH (μM)	121	70	25	n.d.	26

Samples of the isoenzymes were prepared by DEAE-cellulose chromatography of leaf tissue extracts or obtained from dry seed or M9 cell suspension cultures as appropriate.
n.d., Not determined.

Localisation within the cell

Protoplasts isolated from shoot tissue that had been totally submerged for 24 hr were gently ruptured and the organelles separated on a sucrose density gradient as described by Wallsgrove *et al.* [22]. The intactness of each organelle was determined by calculating the percentage of the appropriate marker enzyme that moved down the gradient. Chloroplasts (90%, nitrite reductase), peroxisomes (80%, catalase), and mitochondria (70%, fumarate hydratase) all separated as distinct peaks. However, ADH activity was only detectable at the top of the gradient and there was no evidence of any association with an intact organelle. All three sets of ADH isoenzymes were detected by PAGE in the topmost fractions after centrifugation.

Dissociation and reassociation of isoenzymes in vitro

Set II ADH which had been partially purified by DEAE-cellulose chromatography (but still contained traces of Set I activity), was dissociated in 1 M NaCl by freezing. Following reassociation in low ionic strength buffer, the PAGE banding pattern changed substantially and Set III staining became apparent. However the total recovery of ADH activity as measured spectrophotometrically was very low.

Purification of the Set I isoenzyme

After separation on DEAE-cellulose the Set I isoenzymes were further purified by a series of Hydroxylapatite, Sephacryl S-300 and Phenyl Sepharose chromatography. Final specific activities (calculated as μmol/min/mg protein) were obtained for suspension cultures (41.0), shoot tissue (98.4) and dry seed (404) with purification factors (calculated with reference to the initial crude extract) determined as 46, 729 and 120 respectively. Further attempts to purify the Set I isoenzyme by Blue Sepharose, Matrex Gel Red A and AMP-Sepharose Chromatography or iso-electric focussing were unsuccessful.

The Set I protein from the dry seed when analysed by SDS-PAGE showed the presence of one major band with an M_r of 45 000 and three minor bands.

DISCUSSION

The separation by DEAE-cellulose chromatography of three peaks of ADH activity from extracts of flooded barley roots is consistent with the original observation of Harberd and Edwards [13] that three sets of ADH isoenzymes could be detected by starch gel electrophoresis of comparable extracts. However it is clear from Fig. 1 (bottom) that at least six electrophoretic variants are detectable by PAGE, this pattern is not readily explicable by the simple two-gene model as originally proposed by Harberd and Edwards [13], by analogy with the ADH isoenzyme system in maize [10–12].

The six bands are most conveniently explained by postulating the existence of a third type of ADH monomer, which might arise by expression of a third *Adh* gene or by post translational modification of a proportion of the *Adh* 2 monomers. Harberd and Edwards [23] have recently used PAGE to obtain an ADH banding pattern very similar to that presented here and have favoured the former explanation, suggesting that two of the three *Adh* loci are closely linked.

It could be argued that the three ADH peaks obtained after DEAE-cellulose chromatography or the ADH bands after PAGE are due to artifacts arising in the extraction procedure. The pattern was obtained consistently from other barley tissues (apart from the dry seed) and under a wide range of extraction and storage conditions. The mutant tissue culture M9 however had only Set III activity. The only evidence of an alteration in the pattern was obtained when Set II ADH was dissociated in high salt and allowed to randomly reassociate with the formation of Set III bands. However, the bands of ADH activity observed on PAGE after reassociation were not of equal intensity, which may reflect differences in the specific activity of the different dimers or that some associations are preferred to others. Reassociation of barley and maize ADH subunits has been reported previously [24].

If the existence of three genes for ADH in barley is correct then Set I consists solely of ADH1–ADH1 dimers, Set II consists of ADH1–ADH2 and ADH1–ADH3 dimers and Set III contains ADH2–ADH2, ADH2–ADH3 and ADH3–ADH3 dimers. Despite the heterogenous nature of Sets II and III, the reaction kinetics of the samples conformed well within the Michaelis–Menten model, with double reciprocal plots being linear. Such an observation suggests that there may be little or no functional difference between the monomers coded for by the genes *Adh* 2 and *Adh* 3. There were however obvious differences between the three sets for a range of non-physiological substrates (Table 2).

The presence of isoenzymes in plant tissues often indicates that the same reaction occurs in more than one cellular compartment [8] and many of the enzymes of the glycolytic pathway have been found both in the cytosol and in the plastids of higher plants [25]. However, all of

the forms of the barley ADH were found exclusively in the soluble fraction using a system known to produce a high yield of intact organelles [22]. The similar pH optima exhibited by the isoenzymes substantiated the view that all were located in the same compartment, although the pH optima of 8.7 are unusually high for cytosolic (or even vacuolar) enzymes. No higher plant has yet been found to possess ADH activity associated with an intact organelle [8].

Although Set I ADH was not purified to homogeneity, SDS–PAGE analysis of the extract revealed the presence of a major protein of M_r of 45 000. The apparent M_r of all the native barley ADH isoenzymes is approximately 90 000 further supporting the suggestion of a dimeric enzyme. The barley enzyme is therefore substantially larger than ADH from rice (76 000) [26], pea (60 000) [27], maize (60–74 000) [28] and rape (66 000) [29] but lower than that of wheat (116 000) [30]. The highest specific activity for barley ADH obtained from the dry seed of 404 μmol/min/mg protein is substantially higher than that purified from other plant sources [26–29], but lower than the three sets of isoenzymes purified from wheat embryos [30] The latter isoenzymes, although only purified 11 6–14.6 fold, had final specific activities ranging between 28 700–36 000 μmole/min/mg protein.

EXPERIMENTAL

Plants. Hordeum vulgare cv. Sundance seeds were soaked in aerated distilled water and grown in either trays of EFF compost or a sand/gravel mixture in a constant environment cabinet (20° day/16° night, 12 hr day in a light intensity of $\mu E/m^2/sec$) Flooding was caused by total immersion of the trays in darkened water baths inside the chamber

Suspension cultures were started from tissue isolated from immature endosperms and grown in the medium described by Murashige and Skoog [31] plus 5 mg/litre 2-4-D.

Enzyme assays Enzyme extracts were prepared by grinding powdered tissue frozen in liquid nitrogen in 10 mM Tricine–KOH, pH 8.2, 10% (w/v) glycerol and 0.1% mercopto-ethanol. Crude extracts were desalted on Sephadex G.25 ADH activity was routinely assayed in the direction of ethanol formation by following the acetaldehyde-dependent decrease in absorbance at 340 mM of a reaction mixture containing 100 mM glycine–KOH, pH 8.7, 0 16 mM NADH, 100 mM acetaldehyde and enzyme in a final volume of 1 ml. Catalase, nitrite reductase and fumarate hydratase were assayed as described by Wallsgrove *et al.* [32].

Protein estimations were carried out by the method of Warburg and Christian [33] or the modified dye-binding method of Bearden [34].

Chromatography DEAE-cellulose was equilibrated in 10 mM tricine–KOH, pH 8.2 containing 0.1% mercaptoethanol and 10% (w/v) glycerol. Samples were loaded in, and the column worked, with two volumes of the same buffer. The applied salt gradient was designed to produce a change in KCl concentration of 1 mM per cm column length.

Hydroxylapatite chromatography was carried out in the same buffer in columns of dimensions 30 cm × 11 cm. A phosphate gradient of 1 mM/ml was applied at the same pH, with a flow rate of 10 ml/hr.

Sephacryl S-300 chromatography was carried out in the same buffer as above in columns of dimension 50 cm × 16 mm with a flow rate of 10 ml/hr. Sodium nitrite, haemoglobin, cytochrome *c*, catalase, β-galactosidase and Blue Dextran were used as marker compounds for determining the molecular size.

Phenyl Sepharose was equilibrated in 10 mM Tricine–KOH, pH 8.2, containing 0.1% mercaptoethanol and 200 mM KCl The sample was loaded in, and the column (30 cm × 11 mM) worked with the same buffer. The separation was carried out by a linear gradient of 0–50% (w/v) ethylene glycol and 200–0 mM KCl at a flow rate of 10 ml/hr.

Electrophoresis. Non-denaturing PAGE was carried out as described by Davis [36]. ADH activity was stained by incubating the cut surface of a starch gel or the whole of the polyacrylamide gel in a solution containing 100 mM Tris–HCl, pH 8.8, 0.1 mM NAD, 0.1 mg/ml^{-1} phenazine methosulphate, 0.3 $mg\,ml^{-1}$ nitroblue tetrazolium and 1% ethanol. Control gels were incubated in a mixture without ethanol. Denaturing SDS–PAGE was based on the method of Laemmli [37]. Lysozyme (14 300), β-lactoglobulin (18 400), trypsinogen (24 000), ovalbumin (45 000) and bovine plasma albumin (66 000) were used as M_r markers.

Sucrose density gradient centrifugation A linear gradient of 20–25% (w/v) sucrose in a volume of 38 ml was employed Catalase, myoglobin and β-galactosidase were used as marker proteins and the $S_{20\,w}$ value determined by the method of Martin and Ames [21].

Enzyme localisation. Protoplasts were isolated from barley leaves, and the organelles separated by sucrose gradient centrifugation as originally described by Wallsgrove *et al* [22] and modified in ref. [32].

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