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PURIFICATION AND CHARACTERIZATION OF ALCOHOL DEHYDROGENASE FROM SOYBEAN

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Abstract—Alcohol dehydrogenase from soybean embryos was purified to homogeneity using ion exchange, gel filtration and hydrophobic chromatography. Isoelectric focusing of ADH reveals four isozyme activities with pIs between 5.4 and 6.1. These different forms are all active with ethanol, propanol, butanol, amyl-alcohol and octanol as substrates. They are dimeric enzymes with a M_r of 74000 ± 4000 made of two monomers with a M_r of about 37000 ± 2000.

INTRODUCTION

The second step in the conversion of pyruvate to ethanol is catalysed by alcohol dehydrogenase (ADH; EC 1.1.1.1) in plants subjected to flooding. This pathway provides the NAD^+ necessary to maintain glycolysis under anaerobic conditions. In plants such as maize, tomato and barley, ADH synthesis is induced by anoxia during flooding [1–3]. All ADH isoenzymes are dimeric and in some plants up to three genes code for a series of isoenzymes not all of which are induced by flooding. In tomato, for example [2], the ADH1 isoenzyme is present during germination while ADH2 is only synthesized during anoxia. The close link between development and enzyme activity suggests that plant hormones also control the synthesis of ADH [4–7]. In addition, ADH mutants have been selected by their resistance to allyl-alcohol [8]. The existence of such diverse systems of ADH gene control constitutes therefore an important reason to use ADH for the study of gene regulation in plants. In soya, it has already been demonstrated [5, 6] that the activity of the ADH enzyme is very high at the beginning of germination and decreases to almost zero after 14 days. It is also inducible by 2,4-dichlorophenoxyacetic acid and by anaerobiosis. Under certain conditions the induction can be up to 35-fold (J. P. Thirion, unpublished work). As the first steps in a study of ADH gene regulation in soya and to the cloning of the ADH gene(s), we report here (i) the first purification to homogeneity of the germination-induced ADH from soya and (ii) the expression of at least four very closely related ADH isoenzymes in the germinating seed.

RESULTS

Embryos (*ca* 14 g) were homogenized and centrifuged as described in the Experimental. The supernatant was mixed with Sepharose S. At pH 6.4, ADH does not bind to Sepharose S. Thus its specific activity increased four-fold (Table 1). DEAE-Sephacel chromatography (Fig. 1A) at pH 7.5 eliminated most major proteins and increased the ADH purity by 33.6-fold (Table 1). Hydroxyapatite (Fig. 1B) increased it another 6-fold. At this point in the purification ADH was the only protein visible after Coomassie Blue staining of SDS-PAGE gels. However, silver staining revealed a series of minor contaminants of which the high and low M_r components were subsequently removed by a Sephadex G-100 column (Fig. 2). The last traces of contaminants were eliminated by a second DEAE-Sephacel column (Fig. 1C) eluted with a shallower gradient at pH 7 instead 7.5 followed by a phenyl-Sepharose column which removed any residual hydrophobic proteins. ADH was pure as judged by SDS-PAGE electrophoresis and silver staining (Fig. 2). On non-dissociating PAGE the purified enzyme showed only one band of activity and this band corresponded with the single visible band of protein.

Table 1. Purification of soybean alcohol dehydrogenase

	Total protein (mg)	activity (U/mg)	Specific purification (times)	Yield (%)
Homogenate	2024	0.125	1	100
Sepharose S	448	0.503	4	89
1st DEAE-Sephacel	34	4.20	33.6	58
Hydroxyapatite	4.3	26.17	209	45
Sephadex G-100	1.7	30.05	240	20
2nd DEAE-Sephacel	0.9	37.10	297	13
Phenyl-Sepharose	0.86	38.10	305	13

Physical and biochemical properties

The M_r of the native non-dissociated ADH was determined to be 74000 ± 4000 on Sephadex G-100 while on

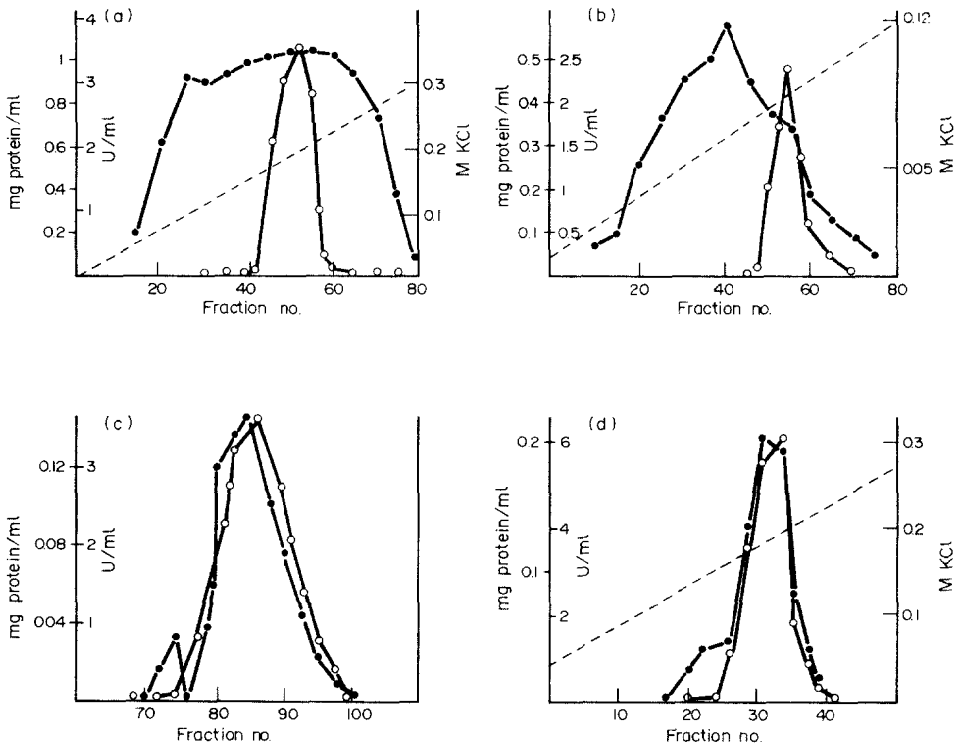

Fig. 1. Elution diagram of four chromatographic steps. (a) DEAE-Sephacel chromatography, (b) hydroxyapatite chromatography, (c) Sephadex G-100 gel filtration, (d) second DEAE-Sephacel chromatography, details are given in Experimental. ●—●, protein; ○—○, ADH activity; – – –, salt gradient.

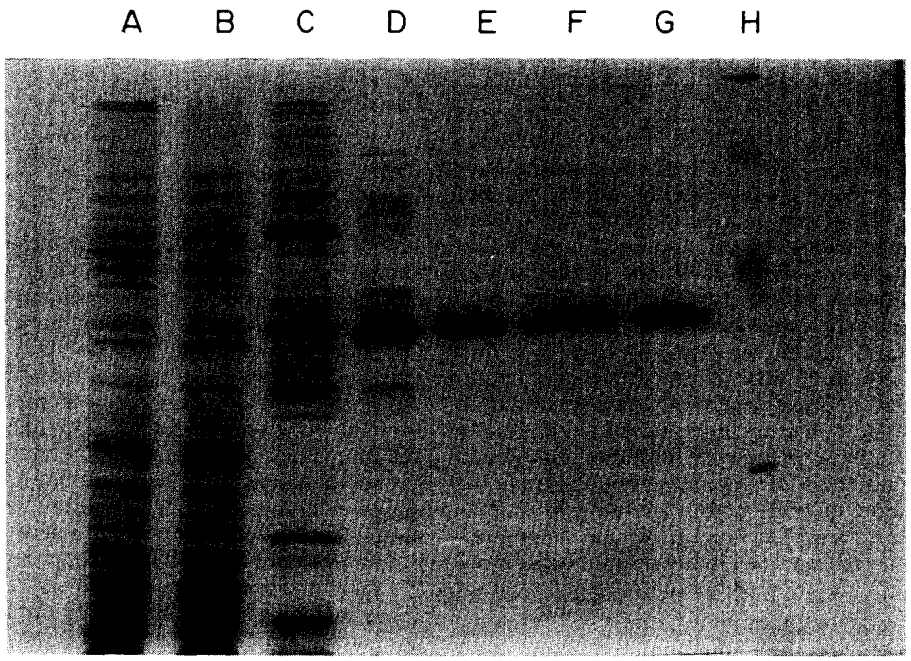

Fig. 2. SDS-PAGE of the combined active fraction after each step of the purification. (A) Supernatant of the homogenate, (B) Sepharose S treatment, (C) DEAE-Sephacel column (D) hydroxyapatite column, (E) gel filtration on Sephadex G-100 column, (F) second DEAE-Sephacel, (G) phenyl-Sepharose, (H) M_r markers: Phosphorylase *b* 94 000, albumin 67 000, ovalbumin 43 000, carbonic anhydrase 30 000, trypsin inhibitor 20 100. The gel was silver-stained as described in Experimental.

SDS gels ADH gave a protein band of M_r 37 000 ± 2000. As expected, soya ADH is a dimeric enzyme. Since many ADHs are classified by their substrate specificity a series of alcohols was tested as substrates for the purified and crude ADH. Both *n*- and *iso*- C_2 to C_8 aliphatic alcohols were substrates, although enzyme activity diminished with increased chain length after C_5. Small unsaturated alcohols such as allyl alcohol were also good substrates. The enzyme was not active with aromatic alcohols and methanol.

The pH optimum in the direction of ethanol formation was 6.7 and the K_m for NADH and aldehyde were 0.100 and 0.180 mM respectively. The pH optimum in the direction of aldehyde formation was between 9 and 10 and the K_m for ethanol was high at 16 mM. The enzyme was inhibited by pyrazole with a K_I of 0.22 mM. Unusual biphasic kinetics were seen for NAD^+ with K_ms of 0.009 and 0.071 mM which suggests that there might be more than one form of the enzyme present.

In order to demonstrate the presence of other forms of ADH both 2D gels and isoelectric focusing (IEF) were carried out with the different ADH fractions. The proteins were separated in 2D gels according to their isoelectric points (1st dimension) and their M_r (2nd dimension). Four closely migrating proteins were detected between pH 5.4 and 6.1. As expected, the M_r of each of these proteins was 37 000. Samples from the different stages of the ADH purification scheme (i.e. homogenate, Sepharose S treatment, first DEAE, hydroxyapatite and phenyl-Sepharose) were run on isoelectric focusing slab gels and stained for ADH activity. Four bands of activity were present in all the preparations in the pH range (5.4–6.1) as they appeared on 2D gel.

DISCUSSION

ADH was purified from soya embryos as part of a project to study its regulation and to clone its gene. Six purification steps were necessary to purify the enzyme to homogeneity (*ca* 300-fold). The yield was 13%. The enzyme has a half-life of only seven days at 4° and this instability is reflected in the decreased yield of the purification steps which took the longest time. Soybean ADH is similar to other ADHs. It is dimeric, has a rather high K_m (16 mM) for ethanol and a high pH optimum (pH 9–10) for the reaction in the direction of aldehyde formation. As expected, the substrate specificity is restricted to aliphatic alcohols between C_2 and C_8, the enzyme is strongly inhibited by pyrazole. In these characteristics the enzyme resembles mammalian ADH1 [9]. However, as with other plant ADHs, this soybean ADH is less stable than mammalian ADH1 and it is inactivated by most precipitation techniques such as ammonium sulphate, alcohol and acetone. Similarly, the enzyme cannot be purified by the AMP-Sepharose columns often used to purify mammalian ADHs, thus the differences probably lie in the cofactor binding site. Our M_r determination of 74 000 and the existence of subunits differs from M_r 53 000 reported previously [14]. The difference between these two results could be due to variations between strains or to artifacts caused by the impurities in the sample of Leblova and Perglerova [14]. We did not observe a change in apparent M_r during purification.

The purified enzyme showed a single band of M_r 37 000 by silver staining on SDS gel electrophoresis, yet the 2-D gels showed four very closely related, but well distinguishable proteins. Similarly, isoelectric focusing of the active enzymes showed four active enzyme bands both in crude and purified preparations. These bands corresponded to the proteins revealed by the 2D gels. The closeness of their pIs explains why they are not noticeable as shoulders on the purification peaks, similarly, they would migrate as one band on non-dissociating PAGE since the pH used for the most efficient separating systems is 1.5 to 4 pH units from their pI. These results and the existence of high and low affinity forms towards NAD^+ suggest that the isoenzymes are the products of different genes and are not due to artifacts of the techniques. However, the physiological roles of the isoenzymes are not yet clear since they all have similar characteristics apart from minor differences in their respective pI and affinity for the coenzyme. It may be that each one is under different regulatory control after germination.

The purification scheme described (coupled to preparative IEF) will permit us to work with purified isoenzymes and to establish the genetic differences between them.

EXPERIMENTAL

Strain and chemicals. Embryos from germinating seed of *Glycine max* L., ("Prize" Variety from Strayer, Hudson, Iowa) soybean were used for purification of ADH enzyme. Fine chemicals, M_r markers, Sephadex G-100, DEAE-Sephacel, hydroxypatite, Sepharose S were purchased from Sigma, BioRad, Pharmacia and Fisher Scientific.

Enzyme assay. ADH activity was determined at 22° in 0.1 M alcohol and in the presence or absence of pyrazole as described previously [5, 9] except that the buffer was 0.1 M Na-glycine (pH 9.8). The activity in the direction of alcohol formation was determined as described [6]. One unit of enzyme activity was defined as the amount of enzyme which catalysed the reduction or oxidation of 1 μmol of NAD/min at 22°. Protein was determined using a dye reagent (BioRad) with bovine serum albumin as standard.

Homogenization. The embryos of soybean seeds (*ca* 300 g) soaked in water for 2 hr were removed and frozen quickly on dry ice. *Ca* 14 g of embryos were homogenized for 5 × 10 sec with 28 ml of buffer A (10 mM K-Pi-citrate, 10 mM 2-mercaptoethanol and 10% glycerol, pH 6.4) with a Polytron homogenisor (Brinkmann Instruments) at 0–4°. The slurry was centrifuged at 10 000 g for 10 min at 4°. The pellets were resuspended in 20 ml of buffer A, homogenized again and centrifuged as above.

Sepharose S chromatography. The above supernatants were pooled and mixed with about 60 ml of a slurry of Sepharose S equilibrated in buffer A. The suspension was agitated gently for 30 min. The slurry was then centrifuged at 200 g for 5 min, resuspended in 60 ml of buffer A and centrifuged again. The supernatants were pooled and used in the next step.

DEAE-Sephacel chromatography. The supernatant was adjusted to pH 7.5 with NaOH and mixed with 50 ml of slurry of DEAE-Sephacel equilibrated in buffer B (10 mM Na-Pi 10 mM 2-mercapto-ethanol and 10% glycerol, pH 7.5). After 5 min the suspension was centrifuged at 200 *g* for 5 min, the gel was washed × 5 in 50 ml of buffer B until no more protein was detected in the supernatant. The DEAE-Sephacel with its adsorbed proteins was layered on top of a 2.6 × 40 cm column of DEAE-Sephacel equilibrated in buffer B. The proteins were eluted at 20 ml/hr with a linear gradient of 0 to 0.3 M KCl in buffer B (360 ml total vol).

Hydroxyapatite chromatography. The active fractions from the DEAE-Sephacel chromatography were pooled and mixed with

50 ml of hydroxyapatite equilibrated with buffer B. After 10 min of stirring the gel was centrifuged at 200 *g* for 5 min. The hydroxyapatite with its adsorbed proteins was resuspended in 50 ml of buffer B and was loaded onto a 2.6 × 40 cm column containing 30 ml hydroxyapatite equilibrated with buffer B. The proteins were eluted with a linear gradient of 10 to 120 mM K-Pi in buffer B in 300 ml total vol at a flow rate of 20 ml/hr.

Sephadex G-100 gel chromatography. The active fractions from the hydroxyapatite were pooled and concentrated from 35 ml down to 2 ml on 'Centricon 30' filters (Amicon). These samples were then loaded onto a 96 × 2.6 cm Sephadex G-100 column. Protein elution was in buffer B at a flow rate of 40 ml/hr.

Second DEAE-Sephacel chromatography. The pooled active fractions (30 ml total) of the Sephadex-G100 column were adjusted to pH 7 with HCl and loaded onto a 10 ml DEAE-Sephacel column (0.8 × 15 cm) in buffer C(10 mM K-Pi, 10 mM 2-mercaptoethanol, 10% glycerol pH 7). The proteins were eluted in buffer C with a linear gradient of 50 to 270 mM KCl (160 ml total vol) at a flow rate of 10 ml/hr.

Phenyl-Sepharose chromatography. The active fractions of the second DEAE-Sephacel column (10 ml total) were adjusted to 0.3 M NaCl and then loaded onto a 10 ml phenyl-Sepharose (0.8 × 15 cm) column equilibrated at 0.3 M NaCl in buffer C. The proteins were eluted in buffer C with a linear decreasing NaCl gradient from 0.3 to 0 M NaCl and increasing polyethylene glycol gradient from 0 to 50% polyethylene glycol as described [10] at a flow rate of 10 ml/hr.

SDS gel electrophoresis. SDS gel electrophoresis was carried out on 10% polyacrylamide gels. The stacking gel concentration was 3%. The sample treatment and buffer composition were those of ref. 11. If the sample vol to be loaded was greater than 50 μl, the proteins were first precipitated with TCA and then redissolved in the treatment buffer (0.125 M Tris–HCl pH 6.8, 4% SDS, 20% glycerol v/v 10% 2-mercaptoethanol v/v). After electrophoresis the gels were washed for 24 hr in MeOH–H_2O and then stained with Ag [12]. Two dimensional gel electrophoresis was carried out according to ref. 13.

Non-dissociating discontinuous PAGE. Stacking gel concentration was 3% in 0.25 M Tris–Pi (pH 6.9) buffer. The concentration of the separating gel was 10% in 0.25 M Tris–Pi (pH 8.5) buffer. The electrode buffer was 40 mM Tris–glycine, for the upper tank and 60 mM Tris–HCl (pH 7.5) for the lower tank.

Isoelectric focusing. The isoelectric focusing was carried out in a 3.5 to 9.5 pH gradient as described in ref. [9]. Gels were stained for ADH activity [5] using 0.3 M of different alcohols. In control experiments, samples were similarly stained but in the presence of 0.1 M pyrazole.

M_r determination. The ADH M_r was determined by gel filtration on a Sephadex-G100 column in buffer B using γ-globulin (160 000), bovine serum albumin (67 000), ovalbumin (43 000), myoglobulin (17 800) and cytochrome *c* (13 000) as standard M_r marketers.

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